WHOLE BLOOD ASSAY ANALYSIS OF IMMUNOSTIMULATORY CONSTITUENTS IN ENVIRONMENTAL PARTICULATE MATTER

by
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Abstract

The respiratory system is continuously exposed to particulate matter (PM) through the inhalation of polluted air. The composition of this PM includes an array of hazardous constituents derived from anthropogenic (e.g. – combustion) and biological sources (biogenic). A fraction of these biogenic agents are non-infectious microbial components that are often capable of engaging innate immune defense mechanisms and stimulating the release of conserved inflammatory mediators. Thus, this biogenic fraction of PM carries a unique capacity to provoke adverse respiratory inflammation in the absence of an infectious challenge or overt physical injury. Accordingly, an increasing epidemiologic evidence-base associates exposure to biogenic PM with the development (and severity) of non-infectious respiratory disorders, such as chronic bronchitis and obstructive lung disease.

However, the integrative methods necessary to evaluate the pro-inflammatory potential of the full matrix of biogenic PM are absent from customary suite of exposure assessment tools. Instead, conventional practice relies on the measurement of individual classes of agents (e.g. – endotoxins) as proxies of pro-inflammatory hazard. This strategy is partly limited by the sheer diversity of stimuli in the environment. The research presented in this dissertation investigates the pro-inflammatory immune responses to PM exposures using novel human whole blood assay (WBA) methods. The human whole blood system carries an extensive array of innate immune defense mechanisms, displays exquisite sensitivity to biologically relevant stimuli, and is amenable to \textit{ex vivo} stimulation with complex mixtures of agents.
This document comprehensively reviews the applicability of the WBA as an exposure assessment tool, and presents two studies in which pro-inflammatory signals of PM were assessed via WBA. The first study used cryopreserved human whole blood to investigate the pro-inflammatory potential of respirable, thoracic, and inhalable PM from broiler poultry housing, where PM is heavily burdened with microbial components. The second study used fresh whole blood from asthmatic and non-asthmatic Peruvian children to evaluate innate immune responses to traffic PM, which has been associated the exacerbation of asthma symptoms. Taken together, this body of research demonstrates the potential of the human WBA to detect hazardous biogenic components in PM, and characterize individual immunologic responses to these complex stimuli.

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Chapter 1
1.1. Introduction

The aims of the research presented in this dissertation are interrelated through the common objective of quantifying the burden of pro-inflammatory constituents in environmental particulate matter (PM). Within the field of exposure science, there is increasing appreciation for the capacity of PM constituents to engage defense mechanisms of the human innate immune system and provoke non-specific airway inflammation (Cherrie et al., 2013; Poole & Romberger, 2012). This “pro-inflammatory potential” of PM represents a complex non-infectious respiratory hazard and has drawn focus towards the immunostimulatory properties of biologically derived (i.e. - biogenic) agents (Liebers et al., 2008; Rylander, 2002).

Many constituents in the biogenic fraction of PM may originate from microbial populations (Douwes et al., 2003). Among these microbial components, Gram-negative bacterial endotoxins are known to be a potent class of pro-inflammatory agents and are the most extensively studied of the biogenic contaminants in PM (Duquenne et al, 2013). However, they are just one of a broad array of microbial constituents that may carry such pro-inflammatory potential. The immunologic recognition of this stimuli results in a complex series of events, which include the release of inflammatory mediators (e.g. – cytokines, chemokines, antimicrobial proteins), recruitment of inflammatory immune cells (e.g. – monocytes, neutrophils), and activation of resident immune cells (Turvey & Broide, 2010).

Although the natural intent of this inflammatory cascade is aimed at combatting pathogenic infection and resolving injury, the inhalation of non-infectious biogenic PM may trigger unwarranted respiratory inflammation (Si-Tahar et al., 2009). Acute
respiratory exposures to biogenic PM are associated with both acute and chronic respiratory health effects that range from reversible lung function decline to chronic obstructive pulmonary disease (COPD) (Lai & Christiani, 2013; Eduard et al., 2009; Donham et al., 2000). The accurate evaluation for the pro-inflammatory hazards of environmental PM remains a formidable challenge and a principal priority for exposure scientists and the greater public health community (HEI, 2010; Pope & Dockery, 2006; Brunekreef & Holgate, 2002).

The research in this dissertation was partly motivated by the methodological limitations that are associated with the analysis of pro-inflammatory PM constituents. Chapter 2 of this document provides an overview of these issues and details the purpose and need for integrated testing methods. The research presented in this dissertation focused on the use of testing approaches that are biologically relevant and model the kinetics of the acute phase inflammatory response following stimulation of the human innate immune system. Human whole blood assay (WBA) methods were chosen as they represent novel in vitro approaches in exposure assessment, and allow for complex mixtures of environmental stimuli to be evaluated in a robust immunologic system.

This dissertation includes two field studies in which human WBA methods were used to assess exposure to environmental PM. The first of these studies is presented in Chapter 3, and is an evaluation for the pro-inflammatory hazards of PM from broiler poultry houses. Poultry house PM is generated almost entirely by the re-suspension of litter containing poultry feces, feeds, bedding materials, feathers and dander (Donham et al., 2000). An abundance of microbial sources contribute to the biogenic composition of this PM, and poultry workers are routinely exposed to high concentrations of aerosolized
particles during their work shifts (Lawniczek-Walczyk et al., 2013; Viegas et al., 2012; Dumas et al., 2011). Inhalation of poultry house PM often results in airway inflammation (Rylander & Carvalheiro, 2006). While endotoxin and non-endotoxin stimuli are thought to mediate this inflammatory activity, the aggregate pro-inflammatory potential of poultry PM is not well understood.

Our investigation into occupational exposures to broiler poultry PM was also motivated by the significant role that the broiler industry holds in the agricultural economy of the Delmarva region, which has one of the highest densities of industrial broiler houses in the United States (US). Poultry production from Delmarva accounts for over five percent of the nine billion broilers that are raised each year in the United States (US) (USDA, 2013). This is a significant volume of production and the long-term sustainability of this industry is partially reliant on the health and safety of its workers, who endure a variety of occupational hazards.

Broiler poultry flocks are housed in facilities that raise high densities of birds to market weight over seven week cycles (MacDonald, 2014). The daily care for broilers is contracted out from industrial hatcheries to small farming operations, who provide the labor and housing facilities (Ollinger et al., 2005). While industrial broiler houses are engineered for efficiency, and may raise flocks of 600,000 or more birds at one time, they foster harsh working conditions (Donham, 2010). An increasing epidemiologic evidence-base suggests that poultry workers, among other laborers in the livestock industry, are at elevated risk for developing a variety non-infectious respiratory disorders (e.g. - chronic bronchitis, COPD) (Eduard et al., 2009; Kirychuk et al., 2003; Donham et al., 2000).
The poultry PM study completed for this dissertation aimed to characterize the burden of stimuli carried by the respirable, thoracic, and inhalable particle size fractions of PM collected from active broiler poultry houses. We collaborated with investigators from the University of Delaware’s Department of Animal and Food Sciences to conduct PM sampling in two of their broiler research facilities. These houses were operated to model conditions in full-scale industrial facilities. For purposes of comparison, PM was sampled from an active dairy cow barn and from ambient air in urban Baltimore City.

A Limulus Amebocyte Lysate (LAL) test was used to quantify the endotoxin content of aqueous PM extracts. The pro-inflammatory potential of this PM was also evaluated based on the capacity of sample extracts to elicit the release of the inflammatory cytokine Interleukin (IL)-1β in a cryopreserved human WBA. For this testing, we pooled peripheral whole blood donations from five health individuals in order to normalize individual immunologic differences before WBA testing. The pro-inflammatory signal of this PM was assessed by size fraction and contrasted against PM sampled from an active dairy cow barn and from ambient air in urban Baltimore City. Further WBA testing was then completed to determine how much of the signal was mediated by insoluble constituents and non-endotoxin stimuli.

The fourth chapter of this dissertation presents a study that used WBA methods to investigate PM exposures of an entirely different nature. This research focused on the capacity of traffic-related ambient PM to stimulate acute phase inflammation from the immune systems of asthmatic and non-asthmatic children. Exposure to ambient PM that is enriched with mobile source combustion constituents is known to stimulate airway inflammation and is increasingly regarded as an environmental risk factor for asthma
(Delfino et al., 2013; Ghio et al., 2012; McCreanor et al., 2007). Correspondingly, the ambient concentrations of PM are higher near major highways (HEI, 2010).

In the context of childhood asthma, increasing residential proximity to highways has been associated with an elevated prevalence of asthma symptoms (McConnell et al., 2006; Venn et al., 2001), decreased lung function (Holguin et al., 2007), and high rates of allergic sensitization (Baumann et al., 2011; Morgenstern et al., 2008). However, aeroallergens do not appear to consistently explain the hazards of this PM for asthmatics (Miller & Peden, 2014). The capacity of this PM to provoke respiratory inflammation is regarded as an important determinant in its potential hazard for susceptible populations since it is capable of transporting over long distances in the environment, and depositing in the sensitive alveolar region of the respiratory tract (Pope & Dockery, 2006).

This study recruited asthmatic and non-asthmatic children from a peri-urban community in Lima, Peru. These participants lived in residences that were either within 75 meters (m) of a major highway in the study area, or at distances of 150 m or more away from it. The respirable fraction of ambient PM was sampled from air in the study area to assess concentrations of PM mass, black carbon, and endotoxin. Peripheral whole blood was collected from asthmatic and non-asthmatic children and fresh blood was stimulated *ex vivo* with endotoxin and aqueous extracts of local PM. WBA release of the cytokine Interleukin (IL)-1β was quantified as a marker of inflammatory response. Participant cytokine responses were assessed as a function of asthma status and the proximity of their household to the study area highway.
1.2. Research Aims

The overall objective of the research in this dissertation was to evaluate the potential of environmental PM to elicit the release of inflammatory mediators from the human immune system. The human whole blood system was chosen as a model for this research and the respective study hypotheses and aims are presented below.

1.2.1. Project 1: Investigate pro-inflammatory potential of PM from the indoor air of active broiler poultry housing.

The first project in this dissertation used a WBA with cryopreserved human whole blood (pooled from multiple healthy donors) to assess the relative immunostimulatory potential of different particle size fractions of PM collected from three distinct environments. The hypotheses tested for this study included:

$H_1$: The burden of bacterial endotoxins and pro-inflammatory stimuli are distributed evenly across the particle size distribution of poultry house PM, when normalized to units of PM mass.

$H_2$: The endotoxin content and proinflammatory potential of poultry house PM is equivalent to that of PM from the indoor air of a dairy cow barn.

$H_3$: The water soluble fraction of constituents in poultry house PM carries the full burden of pro-inflammatory stimuli.

$H_4$: Endotoxins exclusively mediate the proinflammatory signal in poultry house PM.
The following aims were developed to address the study hypotheses:

- Collect time-integrated area samples of respirable, thoracic, and inhalable PM from broiler poultry housing, a dairy barn, and ambient air in Baltimore City.
  - Determine the mass of PM collected over the duration of sampling.
- Evaluate the endotoxin content in the water-soluble fraction of the respirable, thoracic, and inhalable PM samples using a kinetic chromogenic LAL test.
- Prepare aqueous extracts of PM samples from each environment and determine the capacity of each extract to elicit the release of the cytokine Interleukin (IL)-1β from cryopreserved human whole blood, as a marker of pro-inflammatory potential.
  - Assess the pro-inflammatory potential of the PM extracts, with- and without the water-insoluble fraction of PM constituents
  - Assess the pro-inflammatory potential of the PM extract in the presence of an endotoxin inhibitor.

1.2.2. Project 2: Evaluate the effects of traffic PM exposure on peripheral whole blood cytokine responses from asthmatic and non-asthmatic Peruvian children.

Project 2 used a WBA to assess the variation in systemic innate immune responses between asthmatic and non-asthmatics children. The respirable fraction of PM was sampled from ambient air in the study area to determine concentrations of PM mass, black carbon, and endotoxin. Fresh human whole blood from study participants was stimulated ex vivo with a control standard endotoxin and environmental PM from the study area. In this study, the following hypotheses were tested:
H₁: Ambient concentrations of respirable PM and black carbon are higher in the air within 75 meters of the study area highway, in comparison to concentrations in the air at distances of 150 meters or more.

H₂: Peripheral whole blood from asthmatic children releases greater amounts of the cytokine IL-1β, following ex vivo stimulation with endotoxin or PM, than whole blood from non-asthmatic children.

H₃: Peripheral whole blood from children living near the study area highway (75 meters or closer) releases a greater amount of the cytokine IL-1β, following ex vivo stimulation with endotoxin or PM, than whole blood from children living 150 meters or more from the highway.

The following aims were developed to address the study hypotheses:

- Collect 5-day time-integrated samples of respirable PM from ambient air in the study area.
  - Quantify the mass of PM collected over the duration of sampling.
  - Determine the black carbon content of the respirable PM
  - Evaluate the endotoxin content of the water-soluble fraction of constituents from the respirable PM samples.
- Characterize the IL-1β cytokine response following ex vivo stimulation of participant whole blood with endotoxin, PM$_{2.5}$, and respirable PM.
- Determine leukocyte counts for each peripheral whole blood donation to account for variations in blood monocyte counts.
Chapter 2

Methods for Assessing the Pro-Inflammatory Potential of Biogenic Particulate Matter

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Keywords: Endotoxin, Particulate Matter, Whole Blood Assay, Occupational Exposure
2.1. Abstract

The respiratory system is continuously exposed to environmental particulate matter (PM), which often carries non-viable, non-infectious microbial constituents. Many of these agents are capable of stimulating the release of acute phase inflammatory mediators from the innate immune system. The biogenic fraction of this PM carries a unique potential to provoke adverse airway inflammation in the absence of infectious threat. Recurrent exposures are thought to promote the development of chronic respiratory disease and are common in occupational settings. However, the hazards of these biogenic PM exposures are not easily quantified and are often evaluated solely on the basis of endotoxin testing. This single-pollutant testing strategy lacks the biologic relevance and integrative capacity necessary to evaluate the pro-inflammatory potential of the full PM matrix. This review summarizes the applicability of the limulus amebocyte lysate test and human whole blood assay methods for evaluation of the pro-inflammatory hazards of biogenic PM.
2.2. Introduction and Background

The respiratory system is continuously exposed to particulate matter (PM), which is a heterogeneous mixture of constituents that may change significantly over time, and across environments (Davidson et al., 2005). Due to the complexity of sources in the environment, PM is capable of representing a broad range of acute and chronic health hazards, many of which are mediated by inflammation (Ghio et al., 2012; Lai & Christiani, 2013; Liebers et al., 2006; Pope & Dockery, 2006). The accurate evaluation of this pro-inflammatory hazard remains a formidable challenge and a principal priority for exposure scientists and the greater public health community (Pope & Dockery, 2013; Anderson et al., 2012; HEI, 2010).

Customarily, the study of PM-associated health effects has focused on exposures to anthropogenic PM constituents (i.e. - transition metals, black carbon), with emphasis on chronic cardiopulmonary health endpoints (Dockery, 2009; Pope & Dockery, 2006; Riedl & Diaz-Sanchez, 2005; Brunekreef & Holgate, 2002). A secondary focus has been attributed to infectious hazards of pathogenic bioaerosol agents (e.g. - *Legionella pneumophila*, *Mycobacterium tuberculosis*) and allergic responses to aeroallergens (e.g. - house dust mite allergen) (Guarnieri & Balmes, 2014; Matsui, 2014; Diette et al., 2008; Douwes et al., 2003). However, there is an emerging interest in PM constituents that originate from biological sources and carry potential to stimulate respiratory inflammation through non-allergic pathways (Cherrie et al., 2013; Poole & Romberger, 2012).

Environmental populations of microbes are dynamic sources of these “biogenic” constituents, and are capable of imparting a rich mosaic of enzymes, metabolites, genetic
material, and residual structural components (i.e. – endotoxins, lipoproteins, fungal β-glucans) onto the PM matrix (Douwes et al., 2003). Select classes of these microbial motifs represent exogenous danger signals to the human immune system and are known for their capacity to engage innate immune defense mechanisms (Kawai & Akira, 2011). These agents serve as specific ligands to soluble and cell-bound innate immune pattern recognition receptors (PRR) throughout the body, and have been collectively referred to as pathogen-associated molecular patterns (PAMPs) (Takeuchi & Akira, 2010).

The immunologic recognition of PAMPs, such as bacterial endotoxins, results in a complex series of events, which include the release of inflammatory mediators (i.e. – cytokines, chemokines, antimicrobial proteins), recruitment of inflammatory cells (e.g. – monocytes, neutrophils), and activation of resident immune cells (Turvey & Broide, 2010). While the natural intent of this inflammatory cascade is aimed at combating pathogenic infection and resolving injury, there is an increasing appreciation for the capacity of PAMPs, among other biogenic agents, to drive adverse and unnecessary inflammation in the absence of infectious threat or overt physical injury (Si-Tahar et al., 2009). These biogenic agents represent a unique facet of the non-infectious respiratory health hazards associated with environmental PM exposures. Their role in mediating these hazards is not well understood and not easily measured.
2.3. Pro-inflammatory Potential of Biogenic PM Constituents

The inhalation of environmental PM results in the deposition of particles onto the epithelial and mucosal surfaces of the respiratory tract, where they encounter a complex immunologic interface (Heyder, 2004). These surfaces are populated with a diverse suite of innate and adaptive immune defense mechanisms, which continuously survey for threats from the environment and clear particles from the system (Parker & Prince, 2011). The innate immunologic components (e.g. – alveolar macrophages, surfactant proteins) at this interface are critical as they are calibrated to rapidly detect and respond to conserved signals of danger (Hussell & Bell, 2014). Acute inflammatory responses to stimulation from the environment are integral in the mitigation of pathogenic challenges, and are inherently linked to a variety of functions that include immune cell trafficking, increased phagocytosis, and the coordination of the adaptive immune response (Akira et al., 2006).

However, respiratory exposures to bolus or repetitive doses of biogenic PM appear to over-stimulate or exhaust these pathways. The undue inflammation that results from such exposure is believed to disrupt a carefully maintained state of immunologic homeostasis in the respiratory system, resulting in immunopathology (Hussell et al., 2014; Gwyer et al., 2012). Correspondingly, short-term exposures to biogenic PM have been associated with a variety of inflammation-mediated health effects that include reversible declines in pulmonary function, increased mucus production, and dyspnea (Thorn, 2001; Kirkhorn & Garry, 2000). Repetitive biogenic PM exposures are believed to promote non-infectious respiratory diseases such as chronic obstructive pulmonary disease, asthma, and chronic bronchitis (Cherrie et al., 2013; Lai & Christiani, 2013; Eduard et al., 2009; Matheson et al., 2005). These exposures and health effects are both
common for workers in industries such as agriculture, textiles, waste handling, and machining (Dutkiewicz et al., 2011; Heederik et al., 2007; Hoppin et al., 2003).

While the mechanistic understanding for the immunologic hazards of these respiratory exposures is in a constant state of evolution, the capacity to quantify such hazards and accurately depict exposure-response relationships has remained distinctly limited. The integrative testing methods that are necessary to evaluate the total pro-inflammatory potential of the PM matrix remain absent from the customary suite of exposure assessment tools and strategies. Instead, conventional practice has relied on the measurement of individual classes of agents (e.g. – endotoxins) as proxies of pro-inflammatory hazard (Douwes et al., 2003). This approach draws on the same exposure assessment methods that have been in use for the past four decades and is limited, in part, by the sheer diversity of pro-inflammatory agents in the environment.

2.3.1. Bacterial Endotoxins

The pro-inflammatory hazards of biogenic PM are most often appraised on the basis of bacterial endotoxin content. Of the many microbial agents in the environment, endotoxins are widely regarded as the most potent class of immunostimulatory PAMPs in nature (Beutler & Rietschel, 2003). While the terminology for “endotoxin” generically connotes a constitutive, immunotoxic microbial component, it is almost exclusively employed in reference to aggregates of lipopolysaccharide (LPS) molecules from Gram-negative bacteria. For the purposes of the discussion presented here, endotoxin will reference these immunologically active aggregates of LPS.

The cell wall of nearly all Gram-negative bacteria is densely populated with LPS. These amphiphilic, polar molecules are composed of a core polysaccharide (O-antigen)
and a lipid tail (Lipid A), which serves as an anchor into the bacterial cell membrane (Needham & Trent, 2013). In the environment, LPS molecules readily form aggregates that serve as ligands to the MD2/TLR4 innate pattern recognition receptor complex in the mammalian immune system (Kawai & Akira, 2009). Although the endotoxin-TLR4 relationship is well known and extensively researched, it is noteworthy that the TLR4-mediated pathway is not the sole means of endotoxin recognition by immune cells (Kayagaki et al., 2013). Likewise, a variety of environmental ligands (beyond endotoxin) have been identified for TLR4 (Kawai & Akira, 2011).

Due to the ubiquity of Gram-negative bacteria in the environment, endotoxins are commonly present in dusts and airborne PM (Spaan et al., 2006; Simpson, 1999). They are also contaminants of surface waters, and piped water in which biofilms have developed (Keinänen et al., 2004; Muittari et al., 1980). The LPS molecule is uniquely persistent in the environment and shows resistance to degradation induced by changes in pH, temperature, and pressure (Nakata, 1993). This property of LPS allows for concentrations of endotoxins to accumulate in the environment as the result of recurrent LPS shedding by live Gram-negative bacteria and by bacteria death and cell lysis (Mattsby-Baltzer et al., 1991). Thus, endotoxins are distinctive PM constituents, as their resilience allows them to retain pro-inflammatory potential, independent of the lifespan of the parent bacteria, translating to a non-viable biogenic hazard.

While endotoxin is prevalent at low levels in the environment, it is also detected at remarkably high concentrations in conditions that foster bacterial growth (Basinas et al., 2013). Bioaerosol exposures in environments such as livestock housing and cotton processing facilities are routinely associated with a variety of non-infectious respiratory
symptoms (Duquenne et al., 2013; Liebers et al., 2006). Bacterial endotoxins are also associated with the exacerbation of asthma and are considered to be important contaminants of indoor air in the home and in office spaces (Paba et al., 2013; Doreswamy & Peden, 2011). In the medical field, endotoxins are notorious contaminants of pharmaceutical products, medical devices, and injectable drugs, where they are considered one of the primary causal agents in the development of sepsis (Henderson & Wilson, 1996). These clinical hazards have long-served as a principal motivating factor in the scientific research of endotoxins.

For the purposes of environmental exposure assessment, there are a number of pertinent factors that medical research has revealed about the LPS molecule. Notably, the immunologic potency of endotoxin in the human system varies considerably as a function the LPS Lipid A structure (Maeshima & Fernandez, 2013; Dehus et al., 2006). A variety of studies have shown that the mammalian innate immunity discriminates between different structural moieties of LPS as a function of the number and positioning of acyl chains on the Lipid A tail, as well as the pattern of phosphate groups on the molecule (Gutsmann et al., 2010; Dehus et al., 2006; Mathiak et al., 2003). In general, hexaacylated bi-phosphorylated LPS molecules translate to the most potent endotoxins, while fewer acyl chains or removal of phosphate groups is associated with reduced immunotoxicity (DeMarco & Woods, 2011). Evidence also suggests that this potency is dependent on the manner in which endotoxin is released from the bacterial cell and then recognized by the immune system (Mattsby-Baltzer et al., 1991).

Therefore, within the class of Gram-negative LPS endotoxins, the pro-inflammatory potential of these agents varies considerably. This is a facet of endotoxin
testing that has significantly complicated the risk assessment process for environmental exposures. Endotoxin-based research has predominantly been completed using LPS from strains of \textit{Escherichia coli} (\textit{E. coli}) that mimic the potency of the WHO reference standard for endotoxin (purified LPS from \textit{E. coli} 113:H10) (S. Poole et al., 1997). Although these reference and control standard endotoxins are used for purposes of standardization, research is routinely completed using the most potent LPS molecules. This practice is unlikely to represent the heterogeneity of endotoxins in the environment.

This variable potency of endotoxin has been observed across animal models (Schindler et al., 2003; Helander et al., 1982), \textit{in vitro} cellular systems (Stoddard et al., 2010; Dehus et al., 2006), and in the results of human inhalation studies in which aerosolized doses of different endotoxins have been administered to healthy subjects. A review by Thorn (2001) summarizes many of these studies, and research by Möller et al. (2012) and Michel and colleagues (1997) provide examples of supporting evidence that local and systemic inflammation from inhaled endotoxin is dose-dependent and associated with the zone of exposure in the respiratory system. Therefore, it is important to consider the particle sizes with which endotoxins associate in an aerosol, as particle size is a key determinant for the site of particle deposition in the respiratory tract.

Human inhalation studies have assessed exposures of crude extracts of dust from grain (Becker et al., 1999), cotton (Jamison & Lowry, 1986; Cavagna et al., 1969), and livestock facilities (Harting et al., 2012), reporting comparable inflammatory responses to that of purified endotoxin. However, when the collective results of this research are examined, the measured health outcomes (e.g. – pulmonary function, systemic inflammatory markers) do not consistently correlate with the measured endotoxin content.
of the extracts used in the exposure (Poole & Romberger, 2012). This research suggests that endotoxin content does not fully explain the pro-inflammatory potential of environmental dusts and PM. The lack of a robust exposure-response relationship between the endotoxin content in PM and symptoms of respiratory inflammation (e.g.-lung function decline, wheeze) suggests that there are unique elements within the environment and between individual immune systems that contribute to this variability. Although endotoxin is an established pro-inflammatory constituent that frequently burdens PM, environmental exposures to the respiratory system reflect complex mixtures of biogenic agents. The immunologic response to these mixtures of stimuli may not be accurately reflected by the burden or pro-inflammatory potential of any single PM constituent. Consequently, the exposure assessment community has yet to establish endotoxin as a reliable marker of pro-inflammatory potential and no regulatory endotoxin exposure limits exist in the workplace.

The Dutch Expert Committee on Occupational Safety (DECOS), under The Health Council of the Netherlands, has published the most formal occupational exposure guidelines. The DECOS health-based guideline suggests that occupational exposures should not exceed 100 EU/m$^3$ (8-hr time-weighted averages) for endotoxin in the inhalable fraction of PM (DECOS, 2010). This guideline is based on endotoxin exposure studies from Castellan et al. (1987), Smid et al. (1992), and Post et al. (1998).

Castellan and colleagues (1987) investigated changes in the lung function of healthy volunteers who were exposed to varying concentrations of aerosolized cotton dust over six-hour periods. This study used spirometry to evaluate the volume of air released in the first second of a forced expiration ($\text{FEV}_1$) from lungs of study volunteers.
FEV₁ was measured before and after the cotton dust exposure, serving as a standardized measure of small airway function. The results of the study indicated a strong dose-response relationship between the endotoxin content of the dust exposure and cross-shift lung function decline, which was taken as the decrement in FEV₁ that followed the exposure or work shift. The authors of this study reported a No Observable Adverse Effects Level (NOAEL) of 90 EU/m³, which formed the basis for the DECOS guideline.

Smid and colleagues (1992) conducted a cross-sectional study of endotoxin exposure and baseline lung function (FEV₁) in workers from the animal feed industries. Post et al. (1998) followed the same cohort of animal feed workers over a five-year period, assessing endotoxin exposure and the rate of decline in lung function. Based on these studies, the DECOS investigators determined that 40 years of occupational endotoxin exposure at concentrations of 90 EU/m³ or less would increase the normally observed decline in lung function that occurs with age. Therefore, DECOS proposed an occupational endotoxin guideline of 90 EU/m³, or lower, for both short and long-term exposures to prevent accelerated declines in FEV₁. However, the guideline carries no binding authority in the workplace.

2.3.2. Non-Endotoxin Stimuli

In comparison to the field of endotoxin research, significantly fewer investigations have been conducted into the immunotoxicity of non-endotoxin microbial stimuli and little is known about their respective respiratory hazards. Hasiwa and colleagues (2013) published a comprehensive review of these non-endotoxin constituents, drawing on a broad cross-section of studies in which rabbit pyrogen tests, limulus amebocyte lysate (LAL) assays, and human whole blood assays (WBA) were utilized to
assess inflammatory properties of endotoxin and non-endotoxin stimuli. Although the review by Hasiwa and colleagues is geared towards testing for pyrogenic (fever-inducing) contamination in the pharmaceutical industry, much of the discussion is germane to the investigation of biogenic PM exposures and their pro-inflammatory respiratory hazards. Many environments are rich with microbial sources of the same non-endotoxin stimuli discussed by Hasiwa and associates. These sources include Gram-positive bacteria (lipotechooic acids, lipoproteins), fungi (β-glucans, chitins, mannans), virus (dsRNA, unmethylated CpG DNA motifs), and parasites (glycolipids) (Mogensen, 2009; van der Meer et al., 2009; Kikkert et al., 2007; Teixeira et al., 2002).

Non-endotoxin microbial motifs also signal through innate PRRs, and display the capacity to drive the release of non-specific inflammatory mediators in a manner similar to that of endotoxin. Thus, there is an emergent appreciation for the mechanistic basis that supports the contribution of these stimuli to the pro-inflammatory hazards of biogenic PM, since their sources are often equally prevalent to those of endotoxin. Decades of bioaerosol studies employing culture-based testing methods have demonstrated that diverse populations of fungi and bacteria (both Gram-negative and Gram-positive) may be carried by PM (Douwes et al., 2003). More recently, culture-independent DNA sequencing methods have begun to describe these populations at significantly higher order of detail (Arfken et al., 2014; Blais Lecours et al., 2012).

Only a small number of inhalation studies have been conducted in humans to assess immunologic responses to these aerosolized non-endotoxin stimuli. Hoogerwerf and colleagues (2008) investigated the inflammatory effects of purified LPS and lipoteichoic acids (LTA) on the human lung, reporting that both LPS and LTA were
capable of recruiting neutrophils to the airways of study subjects and both induced the release of inflammatory cytokines and chemokines. However, only LPS appear to be capable of activating neutrophils and alveolar macrophages. Meanwhile, Thorn et al. (2001) studied the immunologic effects of inhaled fungal β-glucans in human subjects, reporting that the exposure elicited eosinophilic responses, as opposed to the typical neutrophilic responses that are reported to follow challenge with purified LPS (Michel et al., 2013; Loh et al., 2006). Thus, it appears that LPS, LTA, and β-glucans carry pro-inflammatory potential of somewhat different natures in the human respiratory system.

Additionally, in vitro testing in cellular systems has shown that the human immune system responds differentially to β-glucans that are derived from different sources (Noss, et al., 2013). This lends support towards the notion that broad variations in potency exist within major classes of microbial stimuli. Further complicating the picture is a collection of in vitro evidence, which suggests that the innate immune system’s response to challenge with endotoxin is modulated by the presence of non-endotoxin stimuli. Specific focus has been directed towards co-exposures to endotoxin and (1–3)-β-D-glucans from fungal matter, due to the ubiquity of both constituents in the environment (Kikkert et al., 2007). The innate immune response to mixtures of stimuli is not well understood, and at the time this review was drafted, there were no known inhalation studies that assessed responses to mixtures of PAMPs, with only a few in vitro studies.

Taken together, the results of this body of research suggest that studies of biogenic PM should ensure that fungal and Gram-positive bacterial sources of pro-inflammatory stimuli are accounted for in the evaluation of respiratory hazards. Given the human immune system’s sensitivity to an exquisite array of microbial agents, an
endotoxin-centric focus on biogenic PM exposures is unlikely to provide a full picture of pro-inflammatory potential or hazard. However, nearly all testing for biogenic PM continues to be completed using the horseshoe crab Limulus Amebocyte Lysate (LAL) test, which is a test that is specific to the detection of endotoxin.
2.4. Endotoxin and Non-Endotoxin Testing Methods

2.4.1. Limulus Amebocyte Lysate (LAL) Test

The standard assay for the detection of endotoxin is the LAL test, which is an *in vitro* endotoxin assay that is commonly used across the fields of medicine and environmental health. The LAL test relies on an enzymatic-clotting cascade in the haemolymph of the Atlantic horseshoe crab (*Limulus polyphemus*) to detect endotoxin (Devleeschouwer et al., 1985). Additionally, LAL tests for β-glucans (LAL-g) (Foto et al., 2004) are commercially available, and a Recombinant Factor C (rFC) test has been developed to detect endotoxins based on a recombinant form of the key factor (Factor C) in the clotting cascade of the horseshoe crab’s blood (Thorne et al., 2010). In the field of exposure assessment, the kinetic chromogenic version of the LAL assay is the most frequently employed for its increased sensitivity, although gel-clot and endpoint assays remain in use as well (Paba et al., 2013). The use of different LAL formats has hindered the standardization for endotoxin testing.

It is important to note that LAL-based endotoxin-testing approaches rely on the kinetics of the arthropod immune system, which detects and responds to endotoxins in a manner that is fundamentally different from the mammalian immune system (Gutsmann et al., 2010; Iwanaga, 2007). Accordingly, the sensitivity of the LAL test does not reflect that of the human immune system with regard to endotoxin from different types of bacteria. As a result, this assay may not provide a biologically relevant measure of the human response to endotoxin in environmental samples (Gutsmann et al., 2010; Stoddard et al., 2010; Brandenburg et al., 2009; Dehus et al., 2006).
<table>
<thead>
<tr>
<th>Factor</th>
<th>LAL Testing</th>
<th>WBA Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detect both endotoxin and non-endotoxin</td>
<td><em>Only detects endotoxin</em> (LAL or rFC). No qualitative or quantitative insight into non-endotoxin constituents (with exception of LAL assay specific for β-Glucans).</td>
<td>Intended to provide <em>integrated immunologic response to all relevant stimuli</em> in sample. May measure multiple immunologic markers of immune response from same test.</td>
</tr>
<tr>
<td>Biologically relevant endpoints</td>
<td>LAL test is predicated on <em>horseshoe crab immune system</em>, and the test’s sensitivity to endotoxin is not equivalent to the human immune response.</td>
<td>WBA is reliant on the direct exposure of human primary immune cells to the sample of interest. <em>Test conducted in the species of interest</em>, using a robust biologic system.</td>
</tr>
<tr>
<td>Test sample matrices with insoluble PM</td>
<td>Chromogenic LAL <em>only assesses water-soluble stimuli</em>. Incapable of detecting particle or filter-bound endotoxin.</td>
<td>Responds to both soluble and insoluble stimuli, and is amenable to testing with endotoxin inhibitors, such as Polymyxin B.</td>
</tr>
<tr>
<td>Resistant to sources of assay interference</td>
<td><em>Prone to false positives</em> from agents such as fungal β-Glucans, bacterial DNA, and protease inhibitors.</td>
<td>Since the WBA is an integrated response to all stimuli in a sample, interference is reduced and checked with spiked samples.</td>
</tr>
<tr>
<td>Commercial availability of test reagents</td>
<td>Available through select vendors, although the horseshoe crab population is fragile and negatively impacted by harvesting of crabs for LAL purposes.</td>
<td>Can be completed using widely available commercial reagents. Human whole blood is readily available and blood draws are routine, low risk procedures. No animals involved in testing.</td>
</tr>
<tr>
<td>High throughput capacity &amp; standardization</td>
<td>LAL is capable of high throughput testing on limited basis, using LAL vendor-supplied testing platforms.</td>
<td>Validated for use with pooled, cryopreserved blood. Incubation volumes can be varied and cytokine analysis may be multiplexed.</td>
</tr>
</tbody>
</table>
The limitations of the LAL assay are compounded by its susceptibility to interference from other environmental agents (i.e. - β-glucans, genetic material), and its inability to detect particle-bound endotoxin or endotoxin carried in turbid aqueous samples (Thorne et al., 2010; Kindinger et al., 2005). The preparation of PM samples for LAL testing requires a multi-step extraction process to solubilize endotoxin from the air sample and elute it into an aqueous medium (Spaan et al., 2008; Spaan et al., 2007). This extraction process also creates challenges in the standardization of testing protocols. Despite efforts to standardize LAL testing, previous studies have reported a variety of factors that contribute to variations in test results (Reynolds et al., 2002, 2005; Douwes et al., 1995). These studies show that differences in results are attributed to factors that include the filter extraction medium, air filter material, storage of samples, and the type of LAL used (e.g. - gel-clot, kinetic, endpoint).

LPS molecules have a high affinity for plastic surfaces, and for air sampling, unknown quantities of endotoxin may adhere to air filter membranes. The recovery of endotoxin from these air filter samples is not well defined. Additionally, since the insoluble particles must be removed prior to testing, cell and particle-bound endotoxins are often discarded from the sample via centrifugation prior to testing. The content of endotoxin carried by the insoluble PM fraction is unclear, although Eduard and colleagueus (2004) suggest that the solubility is highly variable (range: 9% - 83%). The impact of variable water solubility may have profound effects on the measured endotoxin content of samples and illustrates the need for strict standardization of testing protocols.

In addition to LAL testing, gas chromatography with mass spectrometry (GC/MS) has also been used in endotoxin studies to characterize the 3-Hydroxy fatty acids from the
Lipid A tail of the LPS molecule (Poole et al., 2010; Saito et al., 2009; Pomorska et al., 2007). GC/MS is also used to assess the content of ergosterol as a marker of fungal contamination and muramic acid as a marker for peptidoglycans (Szponar & Larsson, 2001). While this GC/MS method very finely characterizes the chemical composition of the Lipid A and provides insight towards the variety of Lipid A structures in the sample, it is an expensive method that is also time intensive and requires complicated sample preparation. The results of 3-Hydroxy fatty acid testing via GC/MS provide a poor quantitative measure of exposure and also do not provide results of biologic relevance.

2.4.2. Whole Blood Assay (WBA) Methods

*In vitro* models using human immune cells have been used as an alternative testing method to the LAL for the detection of both endotoxin and non-endotoxin stimuli. Many studies have investigated the immunotoxic properties of PM with monocultures of immune cells from immortalized cell lines (e.g. – THP-1, Mono-Mac-6) or isolated populations of peripheral blood mononuclear cells (PBMCs) (McConnell et al., 2013; Steenhof et al., 2011; Soukup & Becker, 2001). However, only a small number of studies have pursued the use of human whole blood assay (WBA) methods for the evaluation of PM. The WBA model represents a unique approach towards the analysis of complex environmental samples and carries the biologic complexity necessary to holistically assess immunologic responses to mixtures of stimuli.
<table>
<thead>
<tr>
<th>Name</th>
<th>Reference</th>
<th>Blood Incubation</th>
<th>PM Size Fraction</th>
<th>WBA Response</th>
<th># of samples</th>
<th>Sampling Site</th>
<th>LAL Test</th>
<th>Sample Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measurement of air-borne pyrogens by the in vitro pyrogen test (IPT) based on human whole blood cytokine response</td>
<td>Kindinger et al. (2003)</td>
<td>Fresh Blood (individual donors)</td>
<td>Inhalable</td>
<td>IL-1β</td>
<td>N/A</td>
<td>Sheep and pig stables</td>
<td>Y</td>
<td>Whole filters &amp; impinger fluid</td>
</tr>
<tr>
<td>A new method to measure air-borne pyrogens based on human whole blood cytokine response</td>
<td>Kindinger et al. (2005)</td>
<td>Cryo-preserved (pooled)</td>
<td>Total</td>
<td>IL-1β</td>
<td>N/A</td>
<td>Pig &amp; dairy barns, waste recycling</td>
<td>Y</td>
<td>Whole filters &amp; extracts</td>
</tr>
<tr>
<td>Determination of the inflammatory potential of bioaerosols from a duck-fattening unit by using a limulus amebocyte lysate assay and human whole blood cytokine response</td>
<td>Zucker et al. (2006)</td>
<td>Fresh Blood (individual donors)</td>
<td>Inhalable, Impinger fluid</td>
<td>IL-1β</td>
<td>17</td>
<td>Duck Fattening House</td>
<td>Y</td>
<td>Filter Extract &amp; Impinger Fluid</td>
</tr>
<tr>
<td>Standardization of whole blood assay for determination of pyrogenic activity in organic dust samples</td>
<td>Liebers et al. (2009)</td>
<td>Cryo-preserved (pooled)</td>
<td>N/A</td>
<td>IL-1β, IL-6, TNFa, IL-8, MCP-1</td>
<td>30</td>
<td>Grain facility, chicken &amp; pigeon stable</td>
<td>Y</td>
<td>Filter Extracts</td>
</tr>
<tr>
<td>Name</td>
<td>Reference</td>
<td>Blood Incubation</td>
<td>PM Size Fraction</td>
<td>WBA Response</td>
<td># of samples</td>
<td>Sampling Site</td>
<td>LAL Test</td>
<td>Sample Matrix</td>
</tr>
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<tr>
<td>Evaluation of quantification methods of occupational endotoxin exposure</td>
<td>Liebers et al. (2007)</td>
<td>Fresh (individual donors)</td>
<td>Total</td>
<td>IL-1β</td>
<td>55</td>
<td>Swine, duck, rat &amp; mice housing, and an office setting</td>
<td>Y</td>
<td>Filter Extracts (particle free)</td>
</tr>
<tr>
<td>Pyrogenic activity of air to characterize bioaerosol exposure in public buildings: a pilot study</td>
<td>Bernasconi et al. (2010)</td>
<td>Cryo-preserved (pooled)</td>
<td>Total</td>
<td>IL-1β</td>
<td>N/A</td>
<td>Outdoor Air, Indoor Office Air</td>
<td>N</td>
<td>Whole Air Filters</td>
</tr>
<tr>
<td>Assessment of airborne exposure to endotoxin and pyrogenic active dust using electrostatic dustfall collectors (EDCs)</td>
<td>Liebers et al. (2012)</td>
<td>Cryo-preserved (pooled)</td>
<td>EDC (Settled dust)</td>
<td>IL-1β &amp; MCP-1</td>
<td>61</td>
<td>Composting plants &amp; household indoor air</td>
<td>Y</td>
<td>EDC extracts (particle free)</td>
</tr>
<tr>
<td>Concentration of bioaerosols in composting plants using different quantification methods</td>
<td>van Kampen et al. (2014)</td>
<td>Cryo-preserved (pooled)</td>
<td>Inhalable</td>
<td>IL-1β</td>
<td>124</td>
<td>Composting plants</td>
<td>Y</td>
<td>Filter Extracts</td>
</tr>
</tbody>
</table>
The human WBA relies on the *ex vivo* stimulation of diluted, heparinized peripheral whole blood to elicit the release of acute phase cytokines from leukocytes, with the primary response attributed to blood monocytes (Hartung & Wendel, 1995). This cytokine release represents an integrated immunologic response to the cumulative burden of endotoxin and non-endotoxin stimuli in the exposure. In this manner, the WBA model provides a unique immunologic interface in which the PM sample is brought into direct contact with primary immune cells of the peripheral whole blood system. The use of whole blood is important in this test as it maintains the integrity of the whole blood system, including monocytes and an extensive collection of soluble components (e.g. – complement, soluble CD14, LPS-binding protein), which are critical in the detection of PAMPs and the overall function of the system (Hasiwa et al., 2013).

Whole blood is diluted in sterile cell culture medium and incubated with stimuli under physiologic conditions for 18 – 24 hours (Daneshian et al., 2009). Cytokine responses are measured in the supernatant of the whole blood incubation with an enzyme-link immunosorbent assay (ELISA). The WBA methods referenced here are predicated on the human fever response to pyrogenic stimuli (Dinarello, 2004; Hartung & Wendel, 1995), and primarily rely on the release of the non-specific inflammatory cytokines Interleukin (IL) -1β and IL-6 from blood monocytes as a measure of response (Daneshian et al., 2009). These cytokines have been adopted as suitable markers of pro-inflammatory potential as their production is highly conserved and they are released in a dose-dependent manner to stimulation with a variety of PAMPs (Liebers et al., 2009; Deininger et al., 2008; Hermann et al., 2002).
The suggested positive control for this assay is purified LPS from a reference strain of \textit{E. coli}. Incubation of a endotoxin dilution series with PM samples allows for the WBA response to samples to be referenced back against the cytokine response curve generated by the positive controls. These sample responses are then communicated in terms of “equivalent endotoxin units” and represent the quantity of reference endotoxin necessary to generate an pro-inflammatory cytokine response equivalent to that of the sample (Montag et al., 2007). Since PM samples may carry unknown burdens of stimuli, this method represents a novel approach towards communicating the pro-inflammatory potential of PM samples under a standardized metric.

The WBA carries the capacity to detect endotoxin and non-endotoxin stimuli in both liquid and solid samples. A previous study by Kindinger and colleagues (2005) uniquely demonstrated that entire polytetrafluoroethylene air filter samples could be directly incubated in whole blood, circumventing the need to extract particles from the filter and eliminating the potential for losses of stimuli from the original PM matrix. Additionally, the WBA assay has also been shown to work in conjunction with endotoxin inhibitors (e.g. – Polymyxin B) (Rivera-Mariani et al., 2014), anti-TLR antibodies (Punsmann et al., 2013), and novel TLR2 antagonists (Propylene Glycol) (Draing et al., 2008). WBA methods have also been used in research to measure a variety of mediators, including chemokines (Møller et al., 2005), prostaglandins (von Aulock et al., 2003) and lymphokines (Hermann et al., 2003). The assays are amenable to high-throughput methods. Van Dooren and colleagues (2013) have demonstrated that a multiplexed analysis may be completed to assess a broad panel of cytokines and chemokines into the cell-free supernatant of whole blood incubations.
As opposed to the singular readout of the LAL test, an abundance of immunologic data may be extracted from whole blood incubations, which are capable of providing relevant insight into both innate and adaptive human immune responses. Peripheral whole blood is a non-commercial commodity and blood draws are considered to be routine, low risk activities. Heparinized fresh whole blood is reported to remain responsive to stimulation for up to four hours after it has been drawn (Daneshian et al., 2009), and donors may be pre-screened for infection, allergy, or immunologic susceptibilities that may impact the response of the assay. The use of blood from individual donors preserves the immunologic characteristics of the donor whole blood system. Testing blood from different donors against the same stimuli allows for WBA responses between individuals to be contrasted (Noss et al., 2013; Punsmann et al., 2013; Harting et al., 2012).

The WBA has also been validated for use with cryopreserved whole blood that has been pooled from multiple donors. In the context of biogenic PM analysis, this is an important alternative to the use of fresh blood, as it serves to normalize individual differences in the whole blood system (e.g. – monocyte counts) and endows blood with characteristics of a standardized reagent for the assay. Pooled blood may be cryopreserved with a protocol developed by Schindler and colleagues (2004) and stored at -80°C, or colder. Therefore, whole blood incubations can be completed on a schedule that is independent of the blood collection. The cryopreserved WBA may be used with pooled batches of blood to investigate the cytokine response to different types of PM samples or samples from different environments (Schindler et al., 2004). These WBA methods are promising for situational use in exposure assessment, as they provide
integrative testing methods with which to reproducibly evaluate complex sample matrices.

However, WBA methods have only been used for air quality analysis on a limited basis (as summarized in Table 2.2), and considerable work remains to optimize the test for exposure assessment, such that PM samples may be efficiently and reliably evaluated. Similar to LAL-based endotoxin analysis, the increased standardization of WBA testing methods is crucial in order to ensure the integrity and generalizability of results. The heterogeneous composition of whole blood introduces a number of important limitations that must be carefully addressed to accurately evaluate the biogenic fraction of PM on a quantitative basis. In particular, the whole blood system’s sensitivity towards microbial stimuli (e.g. – endotoxin) may vary considerably (both between individuals, and within the same individual over time), and the acute phase cytokine response to this stimulation represents a non-specific marker of inflammatory activity.

While WBA testing with pooled, cryopreserved whole blood provides a mitigation strategy against individual blood donor differences that may contribute to variations in cytokine response, it does not fully eliminate them (Liebers et al., 2009). Monocytes are the primary source of the acute phase cytokines commonly measured in WBA testing, yet the sensitivity of the acute phase response is influenced by a diverse variety of factors that include the dynamic nature of leukocyte sub-populations, atopic sensitization, and circadian rhythms (e.g. – endogenous cortisol levels) (Hermann et al., 2006). These potential sources of immunologic variability should be carefully considered when the WBA is used as an assay to evaluate the pro-inflammatory potential of PM, or when it is used as a test of individual immunologic responsiveness.
The use of appropriate positive and negative controls is necessary to ensure that the assay is free of contamination and interference. To minimize the variation in testing conditions over time, consistency should be maintained in the type of purified LPS used for positive controls, and the source of sterile cell culture media and incubation vessels. The response of the WBA is dependent on many factors, including incubation conditions, assay reagents, and the composition and viability of the blood used in the test.

The pre-screening of donor blood for differential leukocyte counts (e.g. – monocytes, eosinophils) and other potential markers of infection (e.g. – C-Reactive Protein, LPS Binding Protein) may serve to allow for critical compositional factors of whole blood to be controlled for in the experimental design and analysis of data. It is also noted that whole blood carries a variety of endogenous danger-associated molecular patterns (DAMPs), which may synergistically interact with PAMPs and other stimuli to influence the assay response. Namely, extracellular hemoglobin has been shown to potentiate responses to PAMPs such as endotoxin and LTA (Lee & Ding, 2013, Cox et al., 2012). Red blood cell damage during the handling, storage, and incubation of cells may result in false positive responses or inappropriately amplify cytokine responses to environmental samples.

While the whole blood system affords the immunologic complexity to evaluate the burden of stimuli in biogenic PM, the resulting release of acute phase cytokines is a non-specific marker of response. Samples of environmental PM often carry an unknown burden of stimuli and it is difficult to ascertain the primary immunostimulatory constituents in the sample matrix using results from the WBA, alone. Although the use of endotoxin inhibitors (e.g.- Polymyxin B) in the WBA allows for the response to non-
endotoxin stimuli in a sample to be assessed, further compositional analysis of the sample is often necessary to parse out the individual classes of stimuli that likely represent the predominant pro-inflammatory hazards in the sample.

It also must be re-emphasized that the WBA is a test to model the acute phase inflammatory response to stimuli in a sample. The response of the WBA may not directly correlate with the local innate immune response in the respiratory system. Further research utilizing the WBA in human exposure studies is necessary to correlate WBA responses with measures of pulmonary function and respiratory inflammation.
2.5. Discussion

The LAL and WBA methods discussed here both have roles in the evaluation of biogenic PM hazards. While multiple limitations of the LAL test have been highlighted here, the utility of the assay cannot be discounted as it has provided one of the only means for detecting endotoxin in PM samples. In many instances, endotoxin can serve as an important, easily assessed surrogate for a broader biogenic pro-inflammatory signal. However, over three decades of endotoxin-based research has yet to fully explain the respiratory hazards of these biogenic PM exposures. There is both purpose and need to advance the standards for in vitro testing strategies in this field of research and include methods that are both biologically relevant and immunologically sensitive to endotoxin and non-endotoxin stimuli in environmental PM.

Globally, these exposures impact an expansive workforce and carry a significant burden on health care costs, productivity in the workplace, duration of occupational careers, and the personal health and well-being of exposed workers. Numerous occupational sectors are associated with both biogenic PM exposure and chronic respiratory disease environments. These industries include (but are not limited to): agriculture (Spaan et al., 2006), textiles (Lai & Christiani, 2013), food processing (Simon & Duquenne, 2014; Sakwari et al., 2013), lumber milling (Fransman et al., 2003), fiberglass (Milton et al., 1996), and metalworking (Gilbert et al., 2010; Laitinen et al., 1999). PM exposures in these industries are associated with acute conditions that include episodes of wheeze, cough, dyspnea, and cross-shift lung function decline (Andersen et al., 2004; Donham et al., 2000). Workers are also at higher odds to develop permanent
disorders such as COPD, chronic bronchitis, asthma, and hypersensitivity pneumonitis (Eduard et al., 2009; Matheson et al., 2005; Oesmi et al., 1987).

Agricultural occupations are the most commonly associated with high exposures to endotoxin and biogenic PM, and they also share a long history of chronic respiratory illness (Kirkhorn & Garry, 2000). Livestock care and animal husbandry workers appear to bear the highest risks for developing these respiratory diseases (Samadi et al., 2013; Eduard et al., 2009; Hoppin et al., 2003). Workers in industrialized animal production facilities are thought be the most highly exposed to biogenic PM and there is considerable concern for the long-term health implications of these exposures (Heederik et al., 2007). Compositional analysis of PM from animal housing facilities indicates the presence of a variety of potent immunomodulatory agents, including viable microorganisms, non-viable microbial constituents, pollens, animal dander and enzymes, metals, and antibiotics (Biswas et al., 2012; Viegas et al., 2012; Trabue et al., 2010). The respiratory hazards of animal housing environments are underscored by studies in equine and swine facilities, which show that these animals also develop a variety of non-infectious respiratory symptoms and disease that are positively associated with increasing exposures to PM (Theegarten et al., 2008).

Beyond agriculture, the textile industry is similarly burdened by a lengthy history of obstructive respiratory diseases (e.g. – byssinosis), related to chronic exposures to dust derived from raw and processed cotton (Lai & Christiani, 2013). These health effects have been studied in depth since the 1950’s (Furness & Maitland, 1952) and a variety of occupational tasks have been strongly associated with exposure to aerosolized endotoxins and reduced pulmonary function (Kennedy et al., 1987). While the source of endotoxin in
cotton dust has commonly been attributed to bacteria from the *Enterobacter* genus (Rylander & Lundholm, 1978), further microbiological studies have indicated high levels of fungal species, including *Alternatia* and *Cladosporium* (Domelsmith et al., 1988).

Within the manufacturing industry, machining and metalworking occupations are also associated with endotoxin exposure and respiratory symptoms. Industrial processes that employ metal working fluids (MWF) often generate high concentrations of oil mists and aerosols. These lubricants offer abundant sources of nutrients for microbes and often habitually reused in the workplace, which allows for significant microbial populations to develop and impart components, such as endotoxins, into the MFW medium. Exposure studies have report high level of contamination from Gram-positive and Gram-negative bacteria, mycobacterium, and fungi (Fishwick et al., 2005) (Murat et al., 2012).

MFW also often contains biocides, which are added to curb microbial growth (Murat et al., 2012). However, these additives do little to degrade non-viable microbial PAMPs and biocides, themselves, may represent respiratory hazards. Occupational exposure to MWF is associated with the development of acute symptoms such as wheeze and cough, as well as choric effects that include asthma, hypersensitive pneumonitis, and chronic bronchitis (Fornander et al., 2013; Jaakkola et al., 2009; Fishwick et al., 2005). While endotoxins are commonly isolated from MWF aerosols, polymicrobial populations are known to be present in the MFW medium, as well as chemicals and metals.
2.6. Conclusion

Occupational exposures to biogenic PM and respiratory inflammation are common themes across many industries. The biogenic fraction of constituents in PM from the environments often contains a composite of endotoxin and non-endotoxin agents that carry an innate capacity to engage the human immune system and stimulate inflammatory responses. However, the customary single-component (i.e. endotoxin) approach provides limited insight into inhalation risks when diverse arrays of pro-inflammatory stimuli are present. Human WBA methods represents one approach that has displayed a potential to holistically assess complex mixtures of stimuli and provide quantitative results of biologic relevance. While significant work remains to adapt these methods to air quality analysis, they promise to provide a higher order of insight into both the pro-inflammatory potential of biogenic PM and possible individual immunologic susceptibilities to exposure.
Chapter 3

Whole Blood Assay Evaluation of Pro-Inflammatory Stimuli in Broiler Poultry House Particulate Matter

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Keywords: Endotoxin, Broiler Poultry, Whole Blood Assay, Particulate Matter
3.1. Abstract

The inhalation of broiler poultry house particulate matter (PM) often results in airway inflammation and occupational exposure is associated with development of non-infectious respiratory disease in poultry workers. This inflammation is thought to be mediated by both endotoxin and non-endotoxin stimuli, yet the aggregate pro-inflammatory potential of poultry PM is not well understood. This study aimed to evaluate the burden of stimuli in respirable, thoracic, and inhalable particle size fractions of broiler poultry PM. A dairy barn and ambient air in Baltimore City were sampled for comparison. PM was sampled onto non-pyrogenic polytetrafluoroethylene filter membranes and then eluted into a sterile aqueous medium. A Limulus Amebocyte Lysate (LAL) test was used to quantify PM endotoxin content and pro-inflammatory potential was also evaluated based on the capacity of samples to elicit the release of the inflammatory cytokine Interleukin (IL)-1β in a cryopreserved human whole blood assay (WBA). The endotoxin content and pro-inflammatory potential of poultry PM varied over the growth cycle of the flock. This stimulus was predominantly carried in the non-respirable fraction of particles. WBA cytokine release was significantly attenuated by the removal of insoluble particles from extracts, as well as the use of an endotoxin inhibitor. The results of this study demonstrate that poultry house PM carries a complex and dynamic burden of constituents, which are capable of stimulating the release of conserved inflammatory mediators from the human innate immune system.
3.2. Introduction

The United States (US) supports the largest broiler poultry industry in the World, producing close to nine billion broilers each year (USDA, 2013). These broiler flocks are housed in an estimated 29,000 industrial poultry facilities across the US, which raise high densities of birds to market weight over seven week cycles (MacDonald, 2014). Daily care for these flocks is contracted out from industrial hatcheries to small farming operations, who provide labor and housing facilities (Ollinger et al., 2005). While industrial broiler houses are engineered for efficiency and may raise flocks of 600,000 or more birds at one time, they foster harsh working conditions and are associated with exposures to high concentrations of particulate matter (PM) and bacterial endotoxins (Donham, 2010). An increasing epidemiologic evidence-base suggests that livestock workers, including those in poultry houses (broilers and layers), are at increased risk for developing non-infectious respiratory disorders that range from cross-shift lung function decline and dyspnea to chronic bronchitis and chronic obstructive pulmonary disease (Eduard et al., 2009; Kirychuk et al., 2003; Donham et al., 2000).

Broiler poultry house PM is principally generated by the re-suspension of dusts that have been derived from feces, feed, dander, and bedding materials. As a consequence, it carries a heterogeneous mixture of constituents from biologic sources (biogenic), including residual microbial components from Gram-negative bacteria (endotoxins), fungi (β-glucans, mycotoxins), and Gram-positive bacteria (lipoteichoic acids, peptidoglycans) (Lawniczek-Walczyk et al. 2013; Rimac et al., 2010; Viegas et al., 2012). Many of these microbial components are individually capable of engaging defense mechanisms of the innate immune system (e.g. – pattern recognition receptors,
complement), and stimulating non-specific inflammatory activity (Takeuchi & Akira, 2010). Thus, both the concentration and composition of this PM are thought to influence respiratory health hazards for exposed poultry workers.

Bacterial endotoxins are structural components of nearly all Gram-negative bacteria and are considered to be important PM constituents in these exposures. These lipopolysaccharide (LPS) molecules are capable of stimulating airway inflammation and are present at high concentrations in the indoor air of poultry houses (Basinas et al., 2012; Kirychuk et al., 2006). The standard assay for the detection of endotoxin in PM is the Limulus Amebocyte Lysate (LAL) test, which operates on the basis of endotoxin-specific clotting mechanisms derived from the blood of the Atlantic Horseshoe crab (*Limulus polyphemus*) (Iwanaga, 2007). However, the LAL test does not detect non-endotoxin stimuli and responds to endotoxins from different species of Gram-negative bacteria with a lesser degree of sensitivity than the human immune system (Brandenburg et al., 2009). The LAL test is further limited to testing only the water-soluble fraction of stimuli in PM (excluding particle-bound endotoxin) and is prone to interference from common environmental contaminants, such a fungal polysaccharides (Gutsmann et al., 2010; Liebers et al., 2007). Taken together, these limitations suggest that endotoxin measurement via LAL testing may not provide an adequate proxy for the total pro-inflammatory potential of complex biogenic PM, possibly contributing to exposure misclassification.

One alternative method for holistically evaluating the burden of stimuli in the PM matrix is the human whole blood assay (WBA) (Hartung & Wendel, 1995). This method relies on the innate immune defense mechanisms of the human whole blood system (e.g.
– blood monocytes, complement) to detect and respond to stimuli under controlled ex vivo exposures (Daneshian et al., 2009). The release of acute phase pro-inflammatory cytokines, such as Interleukin (IL)-1β and IL-6, is measured as marker of response in the incubation supernatant, using an Enzyme Linked Immunosorbent Assay (ELISA). This method was originally developed to screen for pyrogenic contamination in injectable drugs and medical devices, and has been used on a limited basis for air quality analysis (Liebers et al., 2009; Zucker et al., 2006; Kindinger et al., 2005).

The WBA represents an integrated in vitro testing method, which is sensitive to a broad range of endotoxin and non-endotoxin stimuli, and has proven capable of evaluating complex liquid and solid sample matrices (Hasiwa et al., 2013; Dehus et al., 2006). The assay has also been internationally validated for use with either fresh human whole blood or cryopreserved whole blood, which may be pooled from multiple donors (Schindler et al., 2006; Hoffmann et al., 2005). The use of pooled, cryopreserved blood is attractive for the purposes of air quality analysis and exposure assessment, as this exercise serves to normalize inter-individual differences in the whole blood system (e.g. – monocyte counts, soluble factors). This process creates batches of pooled blood that may be stored at -80°C and thawed as needed for testing over a period of time.

Previous studies by Kindinger et al. (2005) and Liebers and colleagues (2014), (2009), (2007), represent the only published investigations that are known to have used these cryopreserved WBA methods for the explicit analysis of air samples. However, no studies are known to have pursued this approach as means to evaluate the total pro-inflammatory potential of broiler poultry house PM, or to investigate the distribution of stimuli across particle size fractions. We hypothesize that the human WBA, with
cryopreserved blood, provides a unique approach to evaluating and communicating the burden stimuli in size-specific fractions of biogenic PM.

The principal aim of this study was to sample PM from the indoor air of active broiler poultry houses and quantitatively assess the pro-inflammatory burden of endotoxin and non-endotoxin constituents in the respirable, thoracic, and inhalable particle size fractions. The aggregate pro-inflammatory potential of this PM was assessed using a WBA with pooled, cryopreserved human whole blood. Bacterial endotoxin content was quantified with a kinetic chromogenic LAL test. To add context to this analysis, we sought to contrast the pro-inflammatory characteristics of broiler poultry PM with similar PM samples from an active dairy cow barn and the ambient air of Baltimore City.
3.3. Materials and Methods

3.3.1. Sampling Environments and Ethics Approval

Johns Hopkins Bloomberg School of Public Health (JHSPH) Institutional Review Board approved this study (No: 00004693). PM was collected from the indoor air of two broiler poultry houses, a dairy cow barn, and ambient air of Baltimore City. The poultry and dairy facilities utilized for this study were research units operated by investigators from the University of Delaware’s Department of Animal and Food Sciences. Although these facilities were smaller in scale than industrial operations, they were constructed and operated for the purpose of simulating industry practices. Given the management of these houses, we believe the conditions were suitable for the purposes of investigating the pro-inflammatory burden of poultry PM. These facilities also represent model environments in which regular exposure occurs for researchers who tend to the flocks on a daily basis.

A single boiler poultry house (House A, Figures 3.1, 3.2, & 3.3) was sampled on consecutive days in November of 2013, during the final week of a flock’s 42-day growth cycle. The second house (House B, Figure 3.4) was similar in size and construction. House B was sampled four separate times while it contained a single flock of broilers, between September and November of 2014, and once prior to the arrival of the flock. Each poultry house held 2,000 birds at the time of sampling in a floor space of approximately 167 square-meters. Bedding consisted of a mixture of wood chips, feces, feed, and feathers on a soil base. However, bedding in House A was not regularly changed between flocks, while the bedding in House B was replaced just prior to the arrival of the flock sampled. Both of these facilities were equipped with mechanically controlled ventilation and heating, artificial light, and metered feed and water stalls.
Figure 3.1. Exterior of broiler poultry house A

Figure 3.2. Interior of Bay 1 in broiler poultry house A
Figure 3.3. Floor plan of broiler poultry house A

Figure 3.4. Floor plan of broiler poultry house B
Area samples of PM adhering to the respirable, thoracic, and inhalable particle size classifications were collected from each house (ACGIH, 2012). Samplers were placed at an elevation of 1.5 meters (m) above the floor to approximate a worker’s breathing zone. All sampling equipment was tethered to portable stands, which were positioned away from the influence of active exhaust fans and vents. Poultry House A had air intakes along the eves of the roof and four circular exhaust fans (see Figure 3.3). This house had two independently controlled bays that each held 2,000 broilers. Only one bay was sampled for this study. Poultry House B featured three mechanically operated fans and air intake vents positioned along the length of the eves of the roof, at a height of 2.0 m above the facility floor (Figure 3.4). Time-integrated samples were collected over periods of four to six hours. Duplicate or triplicate samples were collected in parallel for each sampling event. Background samples of PM in House B were also collected, which consisted of continuous sampling over a three-day span before the flock arrived.

The dairy barn used for this study was an open-air structure with a concrete floor. The building was situated adjacent to a grain silo and a hay barn, and contained a variety of circulating fans (0.6 – 1.2 m in diameter) for auxiliary ventilation. Approximately 100 dairy cows were housed in individual stalls, with sawdust bedding, at the time of each sampling event. Air samplers were tethered from stands, which were placed in empty animal stalls in the center of the building. Area samples of PM were collected continuously over periods ranging from one to five days. Five independent sets of PM samples were collected from a series of sampling events in December of 2013 and August of 2014. On two occasions, triplicates samples were collected in parallel for each of three size fractions that were investigated.
Ambient PM from Baltimore city was sampled from an urban fourth-floor rooftop location in July of 2014. Samplers were operated continuously over four to five day periods to collect sufficient quantities of PM for analysis. One set of triplicate samples (sampled in parallel) and four additional independent samples (collected in series) for each size fraction were collected from this environment.

3.3.2. PM Sampling

Samples of inhalable PM were collected on pre-weighed non-pyrogenic 25-millimeter (mm) polytetrafluoroethylene (PTFE) filters with a 3-micrometer (µm) pore size (Pall Corporation, Port Washington, NY, USA), using SKC Button Samplers (SKC Model 225-360, SKC Inc., Eighty Four, PA, USA). Similarly, the thoracic particle fraction was sampled using SKC Disposable Parallel Particle Impactors (Catalog # 225-3861, SKC Inc., Eighty Four, PA, USA) and 37 mm PTFE filters with 2 µm pore size. The respirable size fraction of PM was sampled using BGI Triplex cyclones (Model SCC 1.062, BGI, Waltham, MA, USA) and 37 mm PTFE filters.

All samples were collected using BGI 400 Personal Sampling Pumps, which were calibrated to the specified flow rates using a BIOS Drycal primary standard (Bios Intl., Lakewood, CO, USA) and manufacturer supplied calibration caps or a plastic calibration jar (Model 225-111, SKC Inc.). To evaluate quality control during the field sampling, field blanks for all size fractions sampled were included at each sampling event. After field sampling, all filters were transported directly to JHSPH labs, where they were aseptically placed in sterile polystyrene petri dishes and stored in a temperature and humidity controlled room to condition for gravimetric analysis. Filters were weighed before and after each sampling event with a Mettler Toledo XP2U microbalance (Mettler
Toledo, Columbus, OH, USA), which was sensitive to the tenth of a microgram (µg). All samples were allowed to equilibrate in the balance room for a 24-hour period before being weighed. Following gravimetric analysis, these samples were stored in a -20°C freezer until further use.

3.3.3. Preparation of PM Extracts

To extract stimuli and particles from filter membranes, samples were thawed from -20°C at room temperature and aseptically inserted into sterile glass vials (Catalog # SV1020, Allergy Labs, Oklahoma City, OK, USA) under a laminar-flow hood. Filters were then submerged in 10 mL of sterile, pyrogen-free water (PFW) (Catalog #25-055, Corning Cellgro, Manssas, VA, USA), which contained 0.05% Tween20 by volume (Catalog # BP337-500, Thermo Fisher Scientific, Rockville, MD, USA). Each vial was sealed and then placed on an orbital shaker, set to operate at 250 rpm for 60 minutes, at 20°C. Afterwards, samples were sonicated in a room temperature water bath (Aquasonic Model 250HT, VWR International, West Chester, PA, USA) of deionized water for 60 minutes (without heat) at 60 hertz. The samples were then removed from the water bath and mixed vigorously for one minute.

Once the sonicated PM extracts had cooled to room temperature, the extracted material was suspended by vigorously mixing for one minute, once again. One-milliliter aliquots of samples were prepared in sterile 1.5 ml microcentrifuge tubes (Catalog #05-408-129, Thermo Fisher Scientific). One set of aliquots was then centrifuged at 1,000x g for 15 minutes to remove the insoluble particles in the suspension. Seven hundred and fifty microliters of particle-free supernatant from the PM extract was carefully withdrawn.
and transferred to a new set of sterile 1.5 ml microcentrifuge tubes. All 1 ml and 750 µl samples were frozen at -80°C until further testing.

3.3.4. Endotoxin Testing

The endotoxin content of the water-soluble fraction of each PM extract was quantified via a kinetic chromogenic LAL test (Associates of Cape Cod, E. Fallmouth, MA, USA), following the manufacturer’s protocol. Sample extracts were tested in technical duplicate, using a series of 10-fold dilutions ranging from 1:1 to 1:1,000. Ten percent of samples were spiked with control standard endotoxin to a concentration of 0.5 EU/ml, in order to assess for assay inhibition. Purified endotoxin from *Escherichia coli* O113:H10 (Lot #249020, Associates of Cape Cod Inc.) and sterile endotoxin-free water (Catalog #25-055, Corning Cellgro, Manssas, VA, USA) served as positive and negative controls, respectively.

Each assay included a five-point series dilution of the control standard endotoxin (concentrations of 50, 5, 0.5, 0.05, and 0.005 EU/ml). Media blanks and field blanks were included in the extraction and LAL testing as well. The limulus lysate (Pyrochrome® Lot #2041204, Associates of Cape Cod Inc.) was reconstituted with the manufacturer-supplied buffer (Glucashield® Lot #1207034, Associates of Cape Cod Inc.), which served to protect against false positive responses that may result from β-glucans present in the sample matrix. The limit of detection (LOD) for the test was 0.027 EU/ml and samples with endotoxin content below the LOD were assigned a value of ½ the LOD (Adams, 1997).
3.3.5. Whole Blood Collection & Cryopreservation

The WBA used for this study is based on protocols described in detail by Daneshian et al. (2009) and Schindler et al. (2004), (2006). Blood donations were collected from consenting healthy adult participants in the JHSPH community, who were enrolled after they provided an informed oral consent and self-reported to be free from clinically diagnosed allergy, recent or ongoing infections (bacterial, viral, or parasitic), pregnancy, and use of prescribed anti-inflammatory or immunomodulatory drugs. Venous whole blood was collected from participants by a certified phlebotomist, using sterile sodium-heparin blood collection tubes (Catalog #367874, Becton Dickinson, Franklin Lakes, NJ, USA). Random samples of these blood collection tubes were pre-tested to ensure they were free of pyrogenic contamination and did not stimulate the spontaneous release of IL-1β (data not shown here). All blood samples were stored at room temperature (out of direct light), until processing for cryopreservation, which was initiated within one hour of the initial blood draw.

Equal volumes of individual donor blood was pooled into sterile 50 ml polypropylene conical tubes and gently mixed. Cryopreservation-grade Dimethyl Sulfoxide (Catalog #BP231-100, Thermo Fisher Scientific) was then slowly added to a final concentration of 10% (v/v) with constant rotation of the tube. Pooled blood was immediately aliquoted to pre-cooled (0°C) sterile 5 ml and 2 ml cryovials (Catalog #10-500-26/27, Thermo Fisher Scientific), which were then kept in an ice water bath for an additional 15 minutes. The blood was then transferred to a -20°C freezer and cooled for 20 minutes at an approximate rate of 1°C per minute. Lastly, the blood was transferred in
a pre-cooled Styrofoam box to a -80°C freezer for storage. Aliquots were removed and thawed as needed for incubations.

3.3.6. Whole Blood Assay

Aliquots of PM extract were removed from -80°C freezer and thawed at room temperature. Each PM extract was vigorously mixed and serial dilutions were prepared with pyrogen-free water in sterile glass vials. PM extracts (with and without insoluble particles) were incubated with cryopreserved whole blood and cell culture medium in 1.5 ml sterile polypropylene microcentrifuge tubes (Thermo Fisher Scientific). These incubation vessels were first prepared with 100 µl of diluted PM extract and 1 ml of sterile RPMI 1640 (w/L-Glutamine) (Catalog #11875-093, Life Technologies, Grand Island, NY, USA), after which 100 µl of thawed cryopreserved blood was added. A subset of incubations were prepared to include Polymyxin B Sulfate, at a final concentration of 10 µg/ml. Polymyxin B Sulfate is an established endotoxin inhibitor (Xgen Pharmaceuticals, Big Flats, NY, USA) (Cavaillon & Haeffner-Cavaillon, 1986), and pilot tests at this concentration sufficiently inhibited the IL-1β cytokine response to the control standard endotoxin at concentrations up to 10 EU/ml, without inducing cell death. All sample extracts tested with Polymyxin B were diluted to an endotoxin content of 10 EU/ml, or less (as measured by LAL analysis) prior to testing.

To inhibit endotoxins in the sample extracts, Polymyxin B was pre-incubated with the extract and cell culture medium in the incubation vessel at 37°C for 60 minutes. To maintain continuity in incubation conditions, all samples without Polymyxin B were similarly pre-incubated. At the end of the pre-incubation phase, cryopreserved whole blood was removed from the -80°C freezer and rapidly thawed in a 37°C water bath. One
hundred microliters of thawed blood was then added to each 1.5 ml microcentrifuge tube containing the stimuli and RPMI. These incubation vessels were closed, gently inverted twice to mix blood with the cell culture medium, and then placed into a humidified incubator at 37°C and 5% CO₂. After 18 hours, the samples were removed from the incubator and inverted once more to mix cytokines in the solution. These incubations were either aliquoted and immediately frozen at -80°C for future analysis, or centrifuged at 10,000 x g for two minutes and tested immediately via ELISA.

Serial dilutions of the PM extracts were tested in the WBA to determine the lowest extract concentration that elicited an IL-1β response equivalent to a 0.5 EU/ml concentration of endotoxin. These sample responses were carried forward for further quantitative analysis. Ten percent of the PM extracts were spiked with 0.5 EU/ml dilutions of endotoxin to assess for assay inhibition. Purified control standard endotoxin (Associates of Cape Cod Inc., East Fallmouth, MA) served as the positive control for the WBA and was matched to the same batch used for the LAL analysis described above. PFW, PFW and Tween20, and RPMI were included in incubations as negative controls. All samples were incubated in triplicate for this analysis.

3.3.7. Cytokine Measurement

IL-1β release from each whole blood incubation was quantified with an in-house sandwich ELISA, using commercially available antibody pairs against human IL-1β (Catalog # MAB601 and BAF201, R&D Systems, Inc., Minneapolis, MN, USA) and flat-bottom 96-well NUNC™ Maxisorp® ELISA plates (Thermo Fisher Scientific). The cell-free supernatant of all incubations were tested in the ELISA in technical duplicate and results were quantified in picograms (pg) of IL-1β per ml of cell-free supernatant. The
biotinylated secondary antibody was detected with a streptavidin phosphatase enzyme (Catalog #15-30-00, KPL, Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) and BluePhos® Microwell Substrate System (Catalog #50-88-00, KPL). Recombinant IL-1β from R&D Systems (Catalog # 201-LB) served as positive control for the assay. The lower limit of sensitivity of the ELISA was 3.9 pg/ml.

3.3.8. Statistical Analysis

Exploratory data analysis and descriptive statistics were completed using the software package GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). IL-1β cytokine responses from the WBA were converted to units of “Equivalent Endotoxin Units” (EEU) for all statistical analysis, in order to compare LAL and WBA results using comparable units. A 4-PL logistic curve was fit to IL-1β cytokine responses that were developed from dose-response experiments using an eight-point series dilution of endotoxin standards (0.1 EU/ml – 10 EU/ml).

The mean IL-1β responses from triplicate incubations of PM extracts were referenced against the logistic curve-fit to determine the concentration of endotoxin that was necessary to elicit an equivalent IL-1β response. The result provided an estimate of each sample’s potency, relative to that of the endotoxin used as a positive control for both the LAL and WBA testing. For reference, 1 endotoxin unit (EU) is defined as 100 pg of purified LPS from the WHO reference strain of *E. coli* (O113:H10) (Poole et al., 1997). Thus, 1 EU/m³ and 1 EEU/m³ represent a burden of stimuli equivalent to approximately 100 pg of the WHO reference standard endotoxin per cubic meter of air.

Concentrations of endotoxin (EU) and equivalent endotoxin stimuli in the PM extracts were normalized to units of PM mass (EU or EEU/mg) and to units of air
sampled (EU or EEU/m³). Normality of the data was assessed with a Shapiro-Wilk test and non-normal data was log-transformed. Statistical comparisons between groups of samples from different environments or different particle size fractions were completed with un-paired, two-tailed Student’s t-tests. A one-way ANOVA with Dunnett’s post-test was used for multiple comparisons to a reference or control group.

Paired Student’s t-tests was used to assess whole blood incubations that were completed using extracts with and without insoluble particles, and extracts with and without the endotoxin inhibitor. Paired, two-tailed t-tests were used to determine the difference between LAL and WBA tests of the same extract, or WBA tests of extracts with and without insoluble particles, or tests with and without the endotoxin inhibitor. Rank-order Spearman correlations were calculated to assess agreement between the LAL and WBA responses to PM extracts. The normality of the data set used for correlation analyses was assessed with the Shapiro-Wilk test. A p-value = 0.05 was defined at the threshold of statistical significance for all statistical analyses.
3.4. Results

3.4.1. Environmental PM Concentrations

In this study, size-selective PM samplers were used to collect PM from the indoor air of two broiler poultry houses and two comparison environments (a dairy cow barn and Baltimore City). The respirable, thoracic, and inhalable particle size fractions were simultaneously sampled in each environment to evaluate the relative burden of particle mass and stimuli in the air. These particle size classifications represent different fractions of particles that are capable of depositing in specific zones of the respiratory tract (Cherrie & Aitken, 1999). Inhalable PM contains the fraction of airborne particles that are capable of entering the nasal passages and depositing throughout the respiratory tract. The thoracic size fraction is a subset of inhalable PM, and correlates with particles that are capable of depositing in the tracheobronchial region, or further in the peripheral lung. The median aerodynamic equivalent diameter (AED) of these particles is approximately 10 µm. The respirable fraction of particles is limited to those which are sufficiently small in diameter to travel beyond the conducting airways and deposit in the gas exchange regions of the lung. The median AED of respirable PM is four µm.

All PM samples were collected on non-pyrogenic PTFE filter membranes, which were weighed for gravimetric analysis and then processed to evaluate the respective content of endotoxin (using the LAL test), and equivalent endotoxin stimuli (using the WBA). Table 3.1 summarizes the average concentrations of PM mass and stimuli present in the air of each of the three sampling environments. Data for the broiler poultry environment reflects five independent sampling events completed in two houses over the final three weeks of the broiler growth cycle.
As expected, average concentrations for all particle size fractions in the indoor air of the poultry houses were significantly greater than concentrations measured in the open air of the dairy barn and ambient air of Baltimore City. Consequently, these poultry houses also exhibited markedly higher concentrations of endotoxin and equivalent endotoxin stimuli than the other environments. While the respirable fraction of particles comprised 18% of the inhalable poultry PM mass, on average, it represented only 10% of the total inhalable endotoxins there were measured via the LAL testing, and less than 2% of the pro-inflammatory signal from the WBA. Thus, a comparatively small fraction of the inhalable endotoxin and stimuli in poultry house air was capable of traveling into the peripheral regions of the lung. A large amount of this pro-inflammatory stimulus is likely to deposit in the nasopharyngeal region of the respiratory system.

On average, thoracic diameter PM accounted for nearly 67% of mass in the inhalable fraction of PM from the poultry house with, and carried 42% of the respective endotoxin content, and less than 20% of the total pro-inflammatory signal in the WBA. More than half of the inhalable endotoxins, and over 80% of the pro-inflammatory signal of the inhalable poultry PM was associated with particles of extrathoracic diameter. However, there remains a notable burden of the endotoxin and pro-inflammatory stimulus that may be inhaled and deposited on surfaces of the conducting airways, where it is likely to be removed via the mucociliary clearance tract or by cellular phagocytosis.
<table>
<thead>
<tr>
<th></th>
<th>Broiler Poultry House</th>
<th>Dairy Barn</th>
<th>Baltimore City</th>
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</thead>
<tbody>
<tr>
<td><strong>N = 5</strong></td>
<td><strong>N = 5</strong></td>
<td><strong>N = 5</strong></td>
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<tr>
<td><strong>PM Concentration (µg/m³)</strong></td>
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<tr>
<td><em>Inhalable</em></td>
<td>1,587 (927 – 2,719)**</td>
<td>18 (6 - 54)</td>
<td>11 (7 - 20)</td>
</tr>
<tr>
<td><em>Thoracic</em></td>
<td>1,074 (784 – 1,417)**</td>
<td>16 (10 - 26)</td>
<td>12 (11 - 14)</td>
</tr>
<tr>
<td><em>Respirable</em></td>
<td>285 (109 – 745)</td>
<td>10 (6 - 18)</td>
<td>10 (8 - 12)</td>
</tr>
<tr>
<td><strong>Endotoxins (EU/m³)</strong></td>
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<tr>
<td><em>Inhalable</em></td>
<td>1,309 (858 – 1,997)**</td>
<td>13 (5 – 35)**</td>
<td>1.0 (0.3 – 2.8)*</td>
</tr>
<tr>
<td><em>Thoracic</em></td>
<td>548 (411 - 729)**</td>
<td>6 (4 – 10)**</td>
<td>0.4 (0.2 – 1.0)</td>
</tr>
<tr>
<td><em>Respirable</em></td>
<td>129 (74 - 226)</td>
<td>1 (0.8 – 2)</td>
<td>0.3 (0.2 – 0.4)</td>
</tr>
<tr>
<td><strong>Equivalent Endotoxins (EEU/m³)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Inhalable</em></td>
<td>3,317 (1,512 – 7,278)**</td>
<td>18 (13 -27)**</td>
<td>0.3 (0.2 – 0.6)</td>
</tr>
<tr>
<td><em>Thoracic</em></td>
<td>583 (370 - 918)**</td>
<td>15 (7 – 28)***</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td><em>Respirable</em></td>
<td>53 (13 - 216)</td>
<td>3 (1 – 7)</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

- **a** Geometric Mean (GM) of five independent samples with 95% confidence interval (CI)
- **b** One-way ANOVA with Dunnett’s Post-tests used to assess equality of means across size fractions within each environment. Respirable PM was treated as reference group for all comparisons. Statistical significance: *p<0.05, **p<0.01, ***p<0.001 indicates difference from reference group.
- **c** N = 4 for thoracic poultry PM.
In contrast to the broiler poultry environment, over 50% of the mass of PM in the air of the dairy barn was attributed to respirable diameter particles, with the remainder allocated to the thoracic size fraction. In a manner similar to the poultry PM, the burden of endotoxin and equivalent endotoxin stimuli was greatest in the largest particle size fractions. Lastly, PM in the ambient air of Baltimore City consisted almost entirely of respirable particles, which carried a minimal burden of pro-inflammatory stimuli.

While these measures illustrate the differences in potential exposures between environments, broiler poultry houses are dynamic environments, where environmental factors (e.g. - feed, temperature, bird weight and activity) may change significantly over time. Therefore, we sampled PM periodically over the course of a flock’s growth cycle in Poultry House B to investigate the variability in concentrations of PM and pro-inflammatory stimuli over time. This house was sampled while it contained a flock of 2,000 birds and results of the sampling are summarized in Figure 3.5.

In poultry house B the average background concentrations of inhalable, thoracic, and respirable PM were 27 µg/m³, 21 µg/m³, and 19 µg/m³, respectively. Over the course of the flock’s 42-day growth cycle, the indoor air concentrations of PM mass, endotoxin, and equivalent endotoxin stimuli increased significantly and were evident in each of the three particle size fractions sampled. The pro-inflammatory stimuli and particle mass associated with the respirable fraction of PM in the indoor air increased the least of the three particle size fractions we investigated. Together, these results describe the dynamic environmental conditions within the indoor air of broiler poultry houses over the duration of a flock’s growth cycle, and indicate that worker exposures to aerosolized PM and pro-inflammatory stimuli may change significantly as the flock ages. These changing
conditions should be taken into account when assessing occupational exposures in these environments.

Figure 3.5. Indoor air concentrations of PM (A), endotoxins (B), and equivalent endotoxin stimuli (C) increase significantly for each of the respirable, thoracic, and inhalable particle size fractions over the 42-day broiler flock growth cycle.
3.4.2. *Endotoxin & Equivalent Endotoxin Stimuli per Unit Mass of PM*

While concentrations of PM and pro-inflammatory stimuli per unit volume of air in these sampling environments varied considerably, the relative potency of stimuli per unit of PM mass was also considered. Table 3.2 summarizes measures of pro-inflammatory burden in EU/mg and EEU/mg for the respirable, thoracic, and inhalable size fractions of PM from each sampling environment.

On a unit mass basis, the burden of endotoxin and equivalent endotoxin stimuli in poultry and dairy PM were similar in magnitude, yet were distributed differently amongst the size fractions of particles. The pro-inflammatory potential per unit mass of respirable and thoracic dairy PM exceeded that of the poultry PM. The results also suggested that the LAL test underestimated the total pro-inflammatory potential of dairy PM at nearly all size fractions, and underestimated the potency of stimuli in the inhalable poultry PM.

To assess the relative agreement between measures of the LAL test and the WBA, the PM extract data from all environments and size fractions were pooled (N = 45) and a rank-order Spearman correlation coefficient was computed. Spearman’s rho, comparing the LAL-derived EU/mg with the WBA measure of EEU/mg, was calculated to be 0.89 (95% CI: 0.79 – 0.94, p<0.001). To evaluate whether the endotoxin content of PM varies with the growth cycle of the flock, the endotoxin content of samples from Poultry House B were normalized to units of PM mass and are summarized in the Figure 3.6, which depicts the mean and standard deviation of triplicate samples from sampling date. No endotoxin data were available for background levels of thoracic PM.

The endotoxin content per unit mass of this PM varied over time and was greatest at day 22 of the flock’s growth cycle. Endotoxins in the inhalable particle size fraction...
increased by a factor of 12 over the background content during the first three weeks of the flock’s growth cycle (Background 99 EU/mg, Day 22: 1197 EU/mg). Similarly, the endotoxin content of Respirable PM tripled in the first three weeks (Background: 260 EU/mg; Day 22: 793 EU/mg). Surprisingly, no difference in endotoxin content per unit mass of PM was observed between the respirable and thoracic fractions.

Table 3.2. Pro-inflammatory signal, per unit mass PM from poultry house, dairy barn, and urban Baltimore City environments

<table>
<thead>
<tr>
<th>PM Size</th>
<th>Broiler Poultry House</th>
<th>Dairy Barn</th>
<th>Baltimore City</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GM (95% CI)</td>
<td>GM (95% CI)</td>
<td>GM (95% CI)</td>
</tr>
<tr>
<td>Fraction</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>MEU/mg</td>
<td>EEU/mg</td>
<td>MEU/mg</td>
<td>EEU/mg</td>
</tr>
<tr>
<td>Inhalable</td>
<td>801* (592 – 1,083)</td>
<td>726*** (571 - 922)</td>
<td>41 *** (19 - 88)</td>
</tr>
<tr>
<td>Thoracic</td>
<td>514 (371 - 712)</td>
<td>394*** (305 - 508)</td>
<td>34 (16 - 74)</td>
</tr>
<tr>
<td>Respirable</td>
<td>378 (188 - 759)</td>
<td>116 (47 - 285)</td>
<td>19 (13 - 30)</td>
</tr>
</tbody>
</table>

LOD = Limit of Detection

\* Geometric Mean (GM) of five independent samples with 95% confidence interval (CI)

\^ One-way ANOVA and Dunnett’s Post-test were used to assess equality of means for EU/mg & EEU/mg, within environments, with respirable PM as a reference category for multiple comparisons. Statistical significance: * p<0.05, ** p<0.01, ***<0.001 indicate significant difference from reference group.
3.4.3. WBA Response to Particle-free Extracts

One key factor that may influence the results of LAL is the requirement to remove insoluble particles from samples prior to testing, in order to mitigate sources of interference in the assay. Since the WBA carries the flexibility to test samples containing solid particles, we chose to incubate air sample extracts both with (+), and without (-), the insoluble fraction of PM. The three particle size fractions tested here were simultaneously sampled in each environment (Poultry House A and the dairy barn). PM extracts were incubated in triplicate with cryopreserved whole blood and Figure 3.7 summarizes the average burden of stimuli per milligram of PM.

Figure 3.6. Endotoxin content per unit mass of PM in Poultry House B varies over the growth cycle of the flock.
The WBA testing of this PM indicated a qualitative reduction in the pro-inflammatory potential associated with the removal of insoluble PM from nearly all extracts tested. Statistically significant reductions in potency were recorded for particle-free extracts of thoracic PM from both the dairy and poultry environments. At the respirable PM size fraction, the removal of insoluble particles from poultry and dairy samples did not attenuate the respective potencies, suggesting that stimuli in this size fraction may have been predominantly water-soluble. It is also notable that the same trend of increasing pro-inflammatory potential across the respirable, thoracic, and inhalable PM extracts is evident, whether particles were included in the incubation or not.
3.4.4. Endotoxin-inhibition Tests

Next, we sought to investigate how much of the total pro-inflammatory potential of this PM was mediated by endotoxin. Since the inhalable and thoracic PM samples from the poultry house and dairy barn exhibited the highest pro-inflammatory potential, a subset of these extracts was incubated with cryopreserved whole blood and an endotoxin inhibitor (Polymyxin B). Triplicate samples of each particle size fraction were again chosen for this analysis to minimize potential environmental variability. All extracts were incubated in triplicate and IL-1β release was measured following stimulation.

For all samples tested with Polymyxin B, the IL-1β signal was reduced, and a significant signal from non-endotoxin stimuli remained. For PM from poultry house A, the incubation of sample extracts with Polymyxin B suggested that non-endotoxin stimuli accounted for 52% of the total IL-1β-inducing capacity of inhalable PM. Similarly, non-endotoxin stimuli represented 60% of the total IL-1β signal for the thoracic fraction of this poultry PM. In dairy PM, non-endotoxins mediated a lower proportion of the pro-inflammatory signal, accounting for 26% and 21% of the IL-1β response in the inhalable and thoracic fractions, respectively.

For samples from each environment, a strong residual inflammatory cytokine response remained after endotoxin activity was inhibited in the sample. We conducted preliminary testing in our lab to verify that the final concentration of Polymyxin B used for these incubations was sufficient to block response to stimulation with control standard endotoxin at concentrations up to 10 EU/ml. In these tests, endotoxin activity was abrogated without significantly inhibiting IL-1β responses to non-endotoxin stimuli, such
as LTA (data not shown here). Thus, for this testing, all PM extracts were diluted in PFW to endotoxin concentrations less than 10 EU/ml.

Table 3.3. Reductions in pro-inflammatory signal following inhibition of endotoxin in PM extracts with Polymyxin B

<table>
<thead>
<tr>
<th>PM Size</th>
<th>Poultry House (EEU/mg)</th>
<th>Dairy Barn (EEU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>Fraction</td>
<td>PM Extract</td>
<td>PM Extract + Polymyxin B Non-Endotoxin Signal (% of Total)</td>
</tr>
<tr>
<td>PM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhalable (N = 3)</td>
<td>2,950 (587)</td>
<td>1,514** (194)</td>
</tr>
<tr>
<td>Thoracic (N = 3)</td>
<td>492 (52)</td>
<td>293* (28)</td>
</tr>
</tbody>
</table>

*a Mean (SD) of triplicate samples

*b Paired Student’s t-test to assess equality of means for samples tested with and without endotoxin inhibitor

Statistical significance: * p<0.05, ** p<0.01.
3.5. Discussion

The research present here is one of a small number of studies that have used a human WBA to investigate the total pro-inflammatory potential of air samples (Liebers et al., 2009; Zucker et al., 2006; Kindinger et al., 2005) It is the first to use a cryopreserved WBA model to evaluate the pro-inflammatory potential of poultry PM from the inhalable, thoracic, and respirable particle size fractions and contrast it with PM from non-poultry environments. This research also conducted endotoxin testing of this PM with a kinetic chromogenic LAL test. While elements of the study support the utility of endotoxin analysis in exposure assessment, they also highlight key limitations associated with LAL testing that may lead to exposure misclassification in some environments.

The results of this study demonstrate that poultry house PM carries a complex and dynamic burden of constituents, which are capable of stimulating the release of conserved inflammatory mediators from the human innate immune system. Although bacterial endotoxins contribute significantly towards the pro-inflammatory potential of this PM matrix, other non-endotoxin stimuli are present and likely act in concert with endotoxin during exposures to promote respiratory inflammation. The presence of this non-endotoxin stimuli is further supported by a number of poultry house exposure assessment studies, which have characterized the diverse populations of Gram-positive, Gram-negative, and fungal microbes that inhabit these environments and serve as sources of biogenic PM constituents (Lawniczek-Walczyk et al., 2013; Rylander & Carvalheiro, 2006; Viegas et al., 2012). The immunogenic properties of this PM continue to suggest that traditional mass-based exposure metrics are inadequate proxies for pro-inflammatory potential (Cherrie et al., 2013; Poole & Romberger, 2012). Similarly, the quantification
of endotoxins in PM has provided a narrow and incomplete evaluation of total pro-
inflammation potential in agricultural PM (Poole et al., 2010).

The inhalable, thoracic, and respirable PM size fractions were sampled for this study in order to quantify and communicate contaminant concentrations under a framework consistent with standard occupational hygiene practice. In the US, the Occupational Health and Safety Administration (OSHA) enforces workplace exposure limits and has classified poultry PM as “Particles Not Otherwise Regulated” (PNOR). This classification is customarily reserved for dusts considered to be inert and innocuous. PNOR is associated with permissible exposure limits (PEL) of 15 mg/m³ for total dust (roughly equivalent to the inhalable fraction) and 5 mg/m³ for the respirable fraction.

The poultry PM concentrations reported in this study are well below the applicable OSHA PELs and are on the low end of concentrations reported in recent review by Basinas et al. (2013) and a multi-farm study by Kirychuk and colleagues (2006), who assessed PM concentrations in 30 layer and 30 broiler poultry houses in Canada. Collectively, this literature describes average concentrations of inhalable PM in broiler houses within a range of 1.1 mg/m³ to 24.2 mg/m³. Similarly, the respirable fraction of PM is reported at concentrations ranging from 80 µg/m³ to 4.20 mg/m³. Thus, there is considerable variability in conditions across different poultry house environments. In the present study, the average inhalable PM concentration in these poultry houses was 1.59 mg/m³, with respirable PM concentrations were measured at 290 µg/m³. Although the broiler research houses used for this study were significantly smaller in scale that industrial houses, they fostered concentrations of PM that were within the range of that reported from industrial houses.
Donham and colleagues (2000) have proposed specific threshold exposure limits of 2.4 mg/m$^3$ and 160 µg/m$^3$ for occupational exposures to inhalable and respirable poultry PM. These concentrations were interpreted as a threshold at which poultry workers were observed to experience a 3% or greater cross-shift decline in FEV$_1$, a measure of lung function in the small airways. In our study, average concentrations of inhalable PM were below these suggested limits, while respirable PM was measured at concentrations 1.8 times greater than the corresponding threshold. The concentrations of PM at the dairy barn and in Baltimore City, which serves as source of PM for comparison purposes, were at least an order of magnitude lower than that of the poultry house.

LAL analysis of these samples indicated that PM from the dairy barn carried a burden of endotoxin that was similar to that of poultry PM, when normalized to units of PM mass. However, by virtue of the high particle mass concentrations in the air at the respective environments, the poultry house represented markedly higher levels of exposure to pro-inflammatory stimuli. Although there are no formal occupational limits or guidelines for endotoxin exposure in the workplace, threshold exposure limits have been proposed for poultry workers by Donham and colleagues (total dust: 614 EU/m$^3$; respirable PM: 0.32 EU/m$^3$). The Dutch Expert Committee on Occupational Safety (DECOS) has also proposed guidelines of 90 EU/m$^3$ (inhalable dust) for general occupational endotoxin exposures (DECOS, 2013). Notably, the DECOS recommendation does not further differentiate exposure limits by PM size fraction.

The average concentration of inhalable endotoxins in these poultry research houses was over twice the suggested limits of Donham and colleagues, and over 17 times greater than the DECOS limit. Less than half of this inhalable endotoxin was associated
with thoracic particles that could travel into the conducting and peripheral airways. For endotoxin in the respirable fraction of poultry PM, the results of this study exceeded the recommendations of Donham and colleagues by a factor of 400. We also note that this limit was surpassed by endotoxin concentrations at the dairy barn, despite PM mass concentrations that were similar to that of ambient air in Baltimore City.

The influence of animal activity has a pronounced effect on endotoxin content at all particle size fractions. The varied distribution of pro-inflammatory stimuli in these particle size fractions is most evident in the series of sampling events conducted in Poultry House B. Here, the concentration of PM and endotoxin changed significantly over the flock’s growth cycle, and changes were not solely attributable to mass concentrations of PM in the air. The trend was partly associated with a decrease in the endotoxin content per unit mass of this PM, suggesting that influence of Gram-negative bacteria on poultry PM varies over time.

When samples for this study were evaluated as a function of their capacity to elicit the release of IL-1β in the cryopreserved WBA, the respective potencies were quantified in a metric of “equivalent endotoxin units”. Thus, the aggregate pro-inflammatory burden of stimuli in any given PM sample was expressed on a scale relative to the pro-inflammatory potency of a common reference *E. coli* endotoxin. This endotoxin served as a positive control in both the LAL and WBA tests. Results of the WBA testing revealed a number of trends across samples that were generally consistent with the endotoxin analysis, with added insight towards to the pro-inflammatory influences of non-endotoxin stimuli in this PM.
One interesting deviation between the results of the WBA and LAL test was related to the relative difference in pro-inflammatory signal between the respirable, thoracic, and inhalable particle size fractions. To illustrate this point, the LAL-measured endotoxin content of the inhalable poultry PM (801 EU/mg) was twice that of the respirable PM (378 EU/mg) (see Table 3.2). However, the WBA testing indicated the inhalable PM signal (1,910 EEU/mg) was 14 times greater than that of the respirable fraction (138 EEU/mg), for these same samples. Such discrepancies in response may be attributed to a variety of factors, which include: 1) the WBA’s capacity to detect endotoxin with a greater range of sensitivity (Brandenburg et al., 2009; Dehus et al., 2006), 2) to respond to non-endotoxin stimuli (Deininger et al., 2008; Hasiwa et al., 2013), and 3) to test complex mixtures of soluble and insoluble PM constituents (Kindinger et al., 2005).

The contribution of the insoluble particles to the WBA signal was specifically investigated in this study, since their removal is required for endotoxin testing in the kinetic chromogenic LAL. The WBA cytokine response to the inhalable and thoracic PM samples was enhanced when PM extracts were tested with insoluble particles, as opposed to when they were tested after centrifugation. The findings of this study indicate that a non-trivial amount of pro-inflammatory stimuli is removed from the sample prior to LAL analysis. This is a potentially important source of stimuli in biogenic PM exposures as insoluble constituents may be cleared from the respiratory system at a different rate and through different mechanisms than water-soluble constituents (Geiser & Kreyling, 2010).

We also investigated the degree to which endotoxin mediates the pro-inflammatory potential of this PM in the WBA. Polymyxin B was used to inhibit the
activity of endotoxin in PM extracts and significant reductions in WBA signal were evident for all samples tested. Similar findings have been reported by Poole et al. (2010) who stimulate peripheral blood monocytes (PBMC) with dust extracts from a variety agricultural environments (i.e – swine, dairy, grain). In this study, the authors similarly treated extracts with Polymyxin B, and found that it did not completely abrogate the release of TNFα, IL-6, and IL-8 from stimulated PBMC cultures. The residual response was partly attributed to muramic acid (a marker of peptidoglycans) and ergosterol (a marker of fungal biomass). This again illustrates the sources of pro-inflammatory stimuli in this PM that are not captured by standard LAL testing.

However, we also note that when the results of the LAL and WBA analyses are assessed via a non-parametric correlation, there remains a strong agreement between the two assays with regards to the ranking of samples based on pro-inflammatory signal (Spearman’s Rho = 0.89, p<0.001). Liebers et al. (2009) and Zucker et al. (2006) completed similar studies of air samples using both LAL and WBA tests and report similar agreement between assay responses. It should be acknowledged that LAL testing remains a staple of bioaerosol assessment and complements the holistic responses produced by the WBA testing methods.

There are a number of limitations that need to be highlighted. The findings of this study are based on an assessment of two poultry houses. These samples provide insight into the distribution and potency of stimuli in the PM, but largely reflect the environmental conditions of the poultry houses at the time of sampling. In particular, the sampling conducted in poultry house B supports the dynamism of these environments and the potential for worker exposures to vary significantly over time. It is important to
understand this variability and clarify how it affects the key particle size fractions in order to assess how exposures may vary over time, with respect to different regions of the respiratory system. Sampling conducted across time periods or seasons may yield different compositions of PM that translate to greater or lesser measures of endotoxin content and pro-inflammatory potential. A more comprehensive longitudinal assessment is necessary to further evaluate the influences of key environmental factors and the variability of the measures over time.

All samples used for endotoxin and WBA analysis in this study represent area samples of PM, as opposed personal exposure measurements taken directly from breathing zones of workers. The range of PM concentrations to which a poultry worker may be exposed to during the course of a work shift may not be the same at that observed in the stationary time-integrated samples collected for this study. In general, area samples are though to underestimate personal exposures (Cherrie & Aitken, 1999). Lastly, the endotoxin and WBA analysis was conducted on aqueous extracts of stimuli prepared from air filter samples. The efficiency with which stimuli are eluted from air filters into the extraction medium is not well understood and thus it is possible that the stimuli in the extract underestimate the true burden of soluble stimuli. Air filter extraction methods for this study were based on commonly used protocols for endotoxin testing. However, as the WBA analysis of particle-free extracts indicated, cytokine response is enhanced when the entire PM matrix (soluble and insoluble) is tested.

The industrialization of the broiler poultry industry has created harsh working environments where dusts are consistently re-suspended into the air by cycles of animal activity and facility ventilation. Even in the poultry research facilities sampled for this
study, workers are repetitively exposed to this PM. The evaluation of these exposures and worker health should account for the complex pro-inflammatory potential of this PM and the chronic nature of occupational exposure. This study is one of a small number to assess the total pro-inflammatory potential PM with human WBA methods. Future studies are needed to develop health-based exposure metrics using the WBA.
3.6. Conclusions

In conclusion, the findings of this study demonstrate that the quantification of endotoxin, alone, does not capture the total pro-inflammatory potential of PM from the broiler poultry house environment. When normalized to PM mass, the larger particle size fractions carried the greatest burden of endotoxin and the highest capacity to elicit the release of IL-1β from the cryopreserved WBA. Approximately one-half of this pro-inflammatory signal was driven by non-endotoxin stimuli, which were not detected via LAL testing. Additionally, measurable quantities of pro-inflammatory stimuli were associated with insoluble constituents, which are also not captured by the LAL test. Broiler poultry houses are harsh occupational environments with high concentrations of PM that carries a robust pro-inflammatory potential. Although these broiler poultry research houses were smaller than industrial-scale facilities, the indoor air concentrations of PM and endotoxin exceeded health-based guidelines that have been proposed for poultry workers.

Acknowledgements

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Chapter 4

Pro-Inflammatory Effects of Traffic PM on Whole Blood Responses of Asthmatic Peruvian Children

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Keywords: Particulate Matter, Asthma, Endotoxin, Black Carbon, Whole Blood Assay
4.1. Abstract

Exposure to particulate matter (PM) from mobile sources (i.e. – traffic-related PM) stimulates airway inflammation and may promote asthma in children living near major highways. However, it is unclear if asthmatics carry increased susceptibility to traffic PM-induced inflammation. This study used a human whole blood assay (WBA) to investigate traffic-related PM (TRPM) exposure and innate immune responses among asthmatic and non-asthmatic children in a peri-urban neighborhood of Lima, Peru. Asthmatic participants were recruited from residences surrounding a major highway in the study area. Ambient PM (respirable) was sampled at their homes to assess environmental exposures to PM, black carbon, and endotoxin. Peripheral whole blood from 32 asthmatic children and 13 non-asthmatic children was stimulated *ex vivo* with endotoxin and local PM. WBA release of the cytokine Interleukin (IL)-1β was quantified as a marker of inflammatory response. IL-1β responses to stimulation with endotoxin and PM were not associated with asthma status. However, PM$_{2.5}$-stimulated WBA responses were 50% lower for participants living within 75 meters of the study area highway, as compared to subjects living at distances of 150 meters or more. The study results suggest that residential proximity to the highway is associated with an attenuated acute-phase inflammatory cytokine response to TRPM constituents at the systemic level for children in this community.
4.2. Introduction

Asthma is an non-infectious respiratory disease, which is commonly characterized by recurrent episodes of airway inflammation, bronchial hyperreactivity, increased mucus production, and airflow obstruction (Diette et al., 2008; Wills-Karp, 1999). Although the asthmatic disease state is classically associated with atopic phenotypes and sensitization to aeroallergens, it is estimated that only one-half of asthma cases are mediated by eosinophilic responses (Pearce et al., 1999). Consequently, there is increasing interest for the hazards of many non-allergenic environmental agents, which are thought to exacerbate asthma symptoms by stimulating inflammatory activity from defense mechanisms of the innate immune system (Alexis & Carlsten, 2014). These exposures often result in the production of reactive oxygen species and the release of non-specific inflammatory mediators, independent of an allergic response (DeKruyff et al., 2014; Simpson et al., 2008).

Traffic-related particulate matter (TRPM) exposures are known to stimulate acute phase inflammation in exposed airways and are increasingly regarded as environmental risk factors for asthma (Delfino et al., 2013; Ghio et al., 2012; McCreanor et al., 2007). Accordingly, multiple epidemiological studies have reported an increased prevalence of asthma in children who live near major highways, and have associated this elevated risk with chronic exposure to traffic-related pollutants, including PM$_{2.5}$ (particles with aerodynamic equivalent diameters $\leq 2.5$ µm) (McConnell et al., 2006; Venn et al., 2001). Ambient concentrations of PM$_{2.5}$ and black carbon (BC) (a marker of combustion) often remain high within 75 – 100 meters (m) of major highways, and decrease to background levels after distances of 150 – 300 m (Zhu et al., 2002; Hitchins, et al., 2000). The
capacity of these particles to drive respiratory inflammation is regarded as an important determinant of their potential hazard for susceptible populations since they are capable of both transporting over long distances in the environment, and depositing in the sensitive alveolar region of the respiratory system (Pope & Dockery, 2006).

However, the task of evaluating TRPM exposures as a function of their aggregate pro-inflammatory potential (rather than particle mass) is complicated by the inherently heterogeneous composition of anthropogenic, biologic, and crustal elements in the PM matrix (Delfino et al., 2008). Many of these constituents are known to individually stimulate non-specific inflammation, yet a single component approach to these exposure assessments remains common practice. The customary methods provide no insight towards the pro-inflammatory potential of PM constituents that act in concert and often lack the necessary biologic relevance and standardization to accurately detect and contrast exposure-response relationships between individuals. Together, these limitations have contributed to a limited understanding for the relative susceptibility of both asthmatics and non-asthmatic to TRPM-induced inflammation.

Therefore, this study sought to holistically investigate the potential of TRPM to induce non-specific inflammatory immune responses in a cohort of asthmatic and non-asthmatic children from a peri-urban neighborhood of Lima, Peru. To model the interplay between traffic PM exposure and the innate immune responses of these children, we used of a human whole blood assay (WBA). This method allowed for peripheral whole blood from individual participants to be directly challenged, ex vivo, with PM that had been size-selectively sampled from their local environment.
Chronic exposures to traffic pollution (including PM) are pervasive in Lima and dense urbanization has rapidly developed on the periphery of the city. Today, Lima is now an expansive population center with an estimated 8.5 million people. Given that the country of Peru has the highest reported prevalence of asthma of Latin American countries, traffic PM exposures may serve as a significant environmental mediator in the observed asthma morbidity in this population (Mallol et al., 2000).

For this study, the WBA represented a unique field applicable test with which individual immune responses to standard inflammatory agents and complex environmental matrices could both be contrasted in order to determine if systemic susceptibilities were evident between distinct groups of subjects. The human whole blood system was a fitting reaction medium as it carries an array of immunologic defense mechanisms (e.g. - innate pattern recognition receptors, complement) that recognize a wide array of biologically relevant stimuli with exquisite sensitivity (Hasiwa et al., 2013). The robustness of the assay is attributed, in part, to the use of peripheral whole blood, which maintains the physiologic composition of the donor whole blood system (e.g. – leukocyte subpopulations, antibodies, soluble factors), and allows immune cells to respond to stimuli in their evolved cellular and molecular context (Hartung & Wendel, 1995). Stimulation of whole blood results in a complex inflammatory cascade that includes the rapid, dose-dependent release of acute phase mediators (e.g. – TNFα, IL-1β, IL-6) from blood monocytes, increased cellular phagocytic activity, and the production of reactive oxygen species (ROS) (Takeuchi & Akira, 2010; Mogensen, 2009).

To complete this research, asthmatic and non-asthmatics children from the study community donated peripheral whole blood, which was incubated under physiologic
conditions with an aqueous particle extract from local air samples of traffic PM$_{2.5}$ and a reference endotoxin. Respirable PM was sampled from ambient air of asthmatic participant homes in order to evaluate ambient concentrations of PM, black carbon (BC, a marker of combustion), and endotoxin (a potent immunostimulatory molecule from Gram-negative bacteria). The respirable fraction of particle was sampled for this study, as opposed to PM$_{2.5}$, as it more closely encompassed the fraction of particles and stimuli that were likely to deposit in the peripheral airways of the respiratory tract (Cherrie & Aitken, 1999). We quantified the capacity of PM and endotoxin to elicit the release of Interleukin (IL)-1β, a conserved inflammatory cytokine, from donor blood and evaluated these responses as a function of participant asthma status and residential proximity to a major highway in the study area.
4.3. Materials and Methods

4.3.1. Ethics Statement

The Institutional Review Boards of the Johns Hopkins University (JHU) School of Medicine (Baltimore, MD, USA) (Study # CIR00007480) and A.B. PRISMA (Lima, Peru) approved this study. Written informed consent for participation was obtained from the parents or guardians of each participant. It was also assured that participants, themselves, were in agreement to partake in the study.

4.3.2. Study Site & Population

This study was conducted in Pampas de San Juan de Miraflores, a peri-urban neighborhood located 25 kilometers south of central Lima, the capitol and economic center of Peru. The Pampas community encompassed a dense population of approximately 60,000 people and primarily consisted of low-to-middle income households (Robinson et al., 2012). A four-lane paved highway bisected the study area, and small single or double lane collector streets (both paved and unpaved) permeated the surrounding community (see Figure 4.1). This main highway was heavily trafficked by commuter buses, freight trucks, and taxis, which served as the primary sources of combustion pollutants in local ambient air.
Figure 4.1. Study area limits in the neighborhood of Pampas de San Juan de Miraflores in Lima, Peru

Preliminary air sampling in Pampas showed that ambient concentrations of PM$_{2.5}$ and BC were twice as high within 50 m of the highway, as compared to distances of 200 m or more (data not shown here). Baumann and colleagues (2011) conducted epidemiological investigations in this community, and reported that children residing in households within 100 m of the highway in the study area (shown in Figure 4.1) had twice the odds of reporting current asthma symptoms, as compared to children living at distances of 384 m or further.
The present study enrolled three groups of children from Pampas, and recruited participants from the cohort of an on-going longitudinal study that examined the effects of genetic susceptibility and indoor air pollution on childhood asthma in this population. Forty-five participants (ages 11 to 20) entered the study between March and June of 2014, with enrollment limited to one subject per household. This study included 16 asthmatic children from residences within 75 m of the highway ("near-highway" exposure group), 16 asthmatic children from residences that were over 150 m from the road ("background" exposure group), and 13 non-asthmatic adolescent controls from throughout the study area. These buffers from the highway were chosen in accordance with the previous PM sampling conducted in the community and similar buffers that have been employed by studies that include McConnell et al. (2006), Venn et al. (2001), and van Vliet et al. (1997).

The general exclusion criteria for participants in this study included: current pregnancy, bacterial or viral infection in the past week, and diagnosis of a chronic respiratory disease, other than asthma (i.e. – tuberculosis, cystic fibrosis). Participants were defined as moderate-to-severe persistent asthmatics if they reported at least one of the following, in the four weeks prior to administration of the study questionnaire: 1) daily asthma symptoms, 2) nighttime awakenings once a week or more, 3) daily use of salbutamol (or any short-acting beta agonist), 4) some limitation with normal activity, and 5) FEV1/FVC 75-79% (ages 9 – 11) or FEV1/FVC measure of 80-84% (ages 12 or older). Spirometry was performed as previously described by Robinson et al. (2011).

Participant screening for atopy was completed with an Immunocap® Phadiatop in vitro assay and three different allergen panels (animal epidermal and proteins, house dust,
and a mold and yeast mix) (ImmunoCAP 250, ThermoFisher Scientific Inc., Rockville, MD, USA). Serum IgE levels greater than 0.1 kUa/L were interpreted as positive IgE antibody response. Participants with positive results for any of the allergen mixes were considered to be atopic.

4.3.3. PM Sampling

The respirable fraction of airborne PM (50% cut-size of 4 µm) was sampled from the rooftops of participant houses to assess ambient concentrations of particle mass, black carbon, and bacterial endotoxin. PM samples were collected continuously over five-day periods, spanning from Monday to Friday each week, between March and June of 2014. On average, five homes were simultaneously sampled each week and samples were collected from a total of 32 residences.

PM was sampled onto non-pyrogenic 37 mm polytetrafluoroethylene (PTFE) filters (Catalog #66159, Pall Corporation, Port Washington, NY, USA) using BGI Triplex cyclones (BGI, Waltham, MA, USA) and SKC XR5000 sampling pumps (SKC Inc., Eighty Four, PA, USA). Samplers were calibrated to operate at a flow rate of 2.5 liters per minute and sampler flow rates were measured at the beginning and end of the sampling period. Duplicate respirable PM cyclones were set up at each home to operate side-by-side (one pre-weighed sample for gravimetric and BC analysis, and a second for endotoxin and WBA testing). Field blanks and duplicates of these samples were collected at 20% of the homes sampled. Eleven participant homes were sampled concurrently with PEM PM$_{2.5}$ impactors (Catalog #761-203A, SKC Inc) in order to assess for differences in PM mass, BC, and endotoxin content of the respirable and PM$_{2.5}$ particle size fractions.
All filter samples collected for gravimetric analysis were returned to JHU labs, where they were conditioned for 24 hours in a temperature and humidity controlled room and post-weighed on a Mettler Toledo XP2U microbalance (Mettler Toledo, Columbus, OH, USA). Following gravimetric analysis, these filters were analyzed for BC content based on a method previously described by Hansen et al. (1984).

### 4.3.4. Air Filter Extraction

Respirable PM and stimuli were extracted from PTFE filter membranes in a Pampas field lab on the same day that sampling units were returned from households. These filters were removed from samplers under a bench-top hood and aseptically transferred into 12 ml non-pyrogenic glass vials (Catalog #03-391-7D, ThermoFisher Scientific Inc.), using clean, gloved hands and forceps. Ten milliliters of pyrogen-free water (PFW) (Catalog #25-055, Mediatech Inc., Manassas, VA, USA), containing 0.05% Tween20 by volume (Catalog #BP337-500, Thermo Fisher Scientific Inc.) was then pipetted into each vial to fully submerge filters in liquid. These vials were capped, vigorously mixed for one minute, and then sonicated in a room temperature water bath (Model # 97043-964, VWR International, West Chester, PA, USA) for 60 minutes to dislodge particles and stimuli from the filter membrane. The resulting PM extract was used as stimuli for whole blood incubations. The remaining sample eluate was pipetted into sterile cryovials (Catalog #10-500-27, Thermo Fisher Scientific), which were frozen at -20°C and transported back to JHU labs for endotoxin analysis.

### 4.3.5. Endotoxin Analysis

The endotoxin content of air filter extracts was quantified since bacterial endotoxins are common contaminants in environmental PM that are known to induce
airway inflammation and are considered risk factors for asthma (Matsui et al., 2013; Doreswamy & Peden, 2011). Endotoxin testing was completed with a kinetic chromogenic Limulus Amebocyte Lysate (LAL) test (Pyrochrome® Lot #2041204, Associates of Cape Cod Inc, E. Fallmouth, MA, USA), per the manufacturer’s instructions. Briefly, LAL lysate was reconstituted with a manufacture-supplied buffer (Glucashield® Lot #1207034, Associates of Cape Cod Inc.) to block potential false-positive interference from β-glucans in the sample matrix. PM extracts were thawed at room temperature and centrifuged at 1,000 x g for 15 minutes to pellet insoluble particles. Dilutions of the extract supernatant were then tested via LAL in duplicate. Each assay included a five-point series dilution of the control standard endotoxin from Escherichia coli O113:H10 (concentrations of 50, 5, 0.5, 0.05, and 0.005 Endotoxin Units (EU)/ml) (Lot #249021, Associates of Cape Cod Inc.). Media blanks, field blanks, and spiked samples (0.5 EU/ml), were included in the analysis for quality control. The LOD for the test was 0.015 EU/ml and samples with endotoxin content below the LOD were assigned a value of ½ the LOD (Adams, 1997).

4.3.6. Blood Collection

Peripheral whole blood was collected from consenting participants on Sundays, between the hours of six and 10 am. Each participant donated blood only once for this study and a regular time and schedule was maintained for all blood draws, since circadian rhythms affect whole blood cytokine release (Hermann et al., 2006). A certified phlebotomist collected 4 ml of blood into sterile blood collection tubes, which contained 75 USP units of sodium heparin (Catalog #367871, Becton Dickinson, Franklin Lakes, NJ, USA). This batch of blood collection tubes was pre-tested to ensure that it was free of
pyrogenic contamination, which could pre-activate the whole blood system (data not shown here). Heparinized whole blood was added to incubation vials within 1 hour of the blood draw, and blood was kept at room temperature and out of direct light until it was incubated.

EDTA-treated whole blood was also collected from participants for complete blood cell (CBC) counts and a white blood cell (WBC) differential. All CBCs were performed by a local third-party laboratory (Medlab, Lima, Peru). One milliliter of EDTA-treated whole blood from each participant was processed using a Sysmex XE-2100 (Sysmex America, Inc., Mundelein, IL). An average of 32,000 cells were counted for the determination of the WBC differential.

4.3.7. Whole Blood Incubation

Peripheral whole blood from all donors was challenged with two concentrations of *E. coli* endotoxin (10 µg/ml and 100 ng/ml) (Lot #249020, Associates of Cape Cod Inc.), PFW (negative control), and a reference solution of PM$_{2.5}$ particles. This PM$_{2.5}$ extract was derived from ambient air samples in the Pampas community, prior to the start of the study. The extract contained a mass concentration of 60 µg/ml (BC/PM = 12%) and an endotoxin content of 0.5 EU/ml. Asthmatic participants had aliquots of their blood incubated with extracts of ambient respirable PM, which were unique to each participant’s house location. Triplicate incubations of PM$_{2.5}$ and respirable PM were included for 10% of study participants.

Sterile 2 ml cryovials (vendor) were prepared with 1.2 ml of sterile RPMI 1640 + L-Glutamine/HEPES, 300 µl of stimuli (i.e. – endotoxin, PM extract, PFW), and 300 µl of heparinized whole blood. These vials were gently mixed and placed in a thermoblock
incubator, set to operate at 37°C. After 24 hours, the vials of blood were removed from
the incubator, mixed, and then aliquoted to 1.5 ml microcentrifuge tubes. Incubated blood
was immediately frozen at -20°C and transferred to a lab at the University of Cayetano
(Lima, Peru) for storage at -70°C.

4.3.8. Cytokine Analysis

Concentrations of IL-1β were measured in the cell-free supernatant of whole
blood incubations for each study participant. Blood was thawed at room temperature,
mixed briefly, and then centrifuged at 10,000x g for two minutes. The resulting
supernatant was withdrawn and tested in technical triplicate in a sandwich enzyme linked
immunosorbent assay (ELISA), using commercial ELISA kits (Duoset Kit DY201, R&D
Systems, Minneapolis, MN). ELISA analysis was completed per the protocol described
by Daneshian et al. (2009), and the sensitivity of the ELISA was 3.9 pg/ml. Participant
cytokine responses were expressed as nanograms (ng) of IL-1β produced per million
exposed monocytes (ng/10^6 monocytes). IL-1β responses from the WBA analysis were
normalized by participant monocyte counts since this cell population serves as the
primary source of IL-1β in the whole blood system and may vary between individuals
(Boneberg & Hartung, 2002).

4.3.9. Statistical Analysis

Statistical analysis was completed in GraphPad Prism 6.0e (Prism, La Jolla, CA,
USA), and exploratory data analysis was conducted to determine summary statistics for
each group of study covariates. All data were evaluated for normality use a Shapiro-Wilk
test, and lognormal data were transformed prior to evaluation with parametric tests. Un-
paired, two-tailed Student’s t-tests, or non-parametric Mann-Whitney U Tests, were used
to assess between-group differences (e.g. – asthmatics vs. non-asthmatics), with regards to subject characteristics, environmental measures, and WBA responses. Pearson correlation coefficients were calculated for log-transformed data to assess relationships between WBA cytokine response and the respective mass, endotoxin, and BC concentrations of the respirable PM extracts. One-way ANOVAs (with Dunnett’s post test) were used to evaluate the equivalency of mean values between three or more groups, with multiple comparisons to a reference group (non-asthmatics). The p-value threshold for statistical significance was set at 0.05.
4.4. Results

4.4.1. Environmental Concentrations of PM, Black Carbon, & Endotoxin

The respirable fraction of ambient PM was sampled from rooftops of participant homes in Pampas. Between March and June of 2014, ambient concentrations of PM and BC were found to be consistently higher at homes near the highway (distance < 75 m), as compared to background distances (distance > 150 m). The bacterial endotoxin content of ambient respirable PM was low and did not differ by proximity to the highway.

<table>
<thead>
<tr>
<th></th>
<th>Near-Highway (^1) (N = 16)</th>
<th>Background (^1) (N = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respirable PM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\mu g/m^3)</td>
<td>27.2 (24.2 – 30.6)*</td>
<td>23.6 (21.0 – 26.6)</td>
</tr>
<tr>
<td><strong>Black Carbon</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\mu g/m^3)</td>
<td>8.4 (7.4 – 9.6)**</td>
<td>5.8 (5.1 – 6.6)</td>
</tr>
<tr>
<td>BC/PM (%)</td>
<td>31% (27% – 34%)**</td>
<td>23% (21% - 25%)</td>
</tr>
<tr>
<td><strong>Endotoxin(^3)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EU/m^3</td>
<td>0.07 (0.04 – 0.13)</td>
<td>0.08 (0.05 – 0.15)</td>
</tr>
<tr>
<td>EU/mg</td>
<td>2.7 (1.4 – 5.2)</td>
<td>3.4 (1.8 – 6.3)</td>
</tr>
<tr>
<td>EU/ml (extract)</td>
<td>0.13 (0.08 – 0.21)</td>
<td>0.19 (0.13 – 0.29)</td>
</tr>
</tbody>
</table>

\(^1\) Near-Highway: residence < 75 m from highway; Background group: residence > 150 m from highway

\(^2\) Statistical significance: * \(p<0.05\), ** \(p<0.01\), *** \(p<0.001\); Un-paired, two-tailed Student’s t test

\(^3\) Endotoxin sample sizes: Near-Highway group (N = 15); Background group (N = 13). Endotoxin data were missing for samples from four participant homes.
The respirable and PM$_{2.5}$ particle size fractions were simultaneously sampled at 11 homes in the study area. Figure 4.2 summarizes the relationship between the average concentrations of this PM in ambient air near the highway and at background distances. Between sampling zones (near-highway vs. background), only the respirable fraction of PM and BC differed by proximity to the highway. A paired, two-way Student’s t-test was used to assess differences between particle sizes fractions within sampling zones No differences in PM mass concentrations or BC content were found, suggesting that the preponderance of respirable particles in the air were in the PM$_{2.5}$ size fraction. However, ambient concentrations of respirable PM and BC remained higher at sampling sites near the highway, as was reported for the analysis of full set of respirable PM samples.

Figure 4.2. Respirable and PM$_{2.5}$ fractions of ambient PM and BC in the study area.

1 Near-Highway: residence < 75m from road
2 Background: residence > 150m from road
3 Data represent median and range
4 Statistical Significance: * p<0.05; un-paired t-test
4.4.2. Study Population Characteristics

Descriptive statistics for study participants are presented in Table 4.2. Asthmatic subjects in this study were predominantly male, while the majority of controls were female. A high prevalence of atopy was reported across all participants, regardless of asthma status. No difference in pre-bronchodilator FEV\textsubscript{1} and FVC, or predicted-FEV\textsubscript{1}, was measured between asthmatic and non-asthmatics groups. On average, asthmatics were found to have an 8% lower baseline FEV\textsubscript{1}/FVC (un-paired Student’s t test, p <0.05).

<table>
<thead>
<tr>
<th></th>
<th>Asthmatics (N = 32)</th>
<th>Controls (N = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years [median (range)]</td>
<td>15 (11 - 20)</td>
<td>14 (11 - 17)</td>
</tr>
<tr>
<td>Sex, % female</td>
<td>22%</td>
<td>69%</td>
</tr>
<tr>
<td>Atopy, %(^1)</td>
<td>70%</td>
<td>80%</td>
</tr>
<tr>
<td>BMI, kg/m(^2) [median (range)]</td>
<td>22.2 (16.5 – 33.0)</td>
<td>23.5 (17.7 – 30.7)</td>
</tr>
<tr>
<td>FVC, L [median (range)]</td>
<td>3.7 (2.2 – 6.4)</td>
<td>3.5 (2.1 – 6.0)</td>
</tr>
<tr>
<td>FEV\textsubscript{1}, L [median (range)]</td>
<td>3.2 (1.7 – 4.9)</td>
<td>3.2 (1.9 – 5.1)</td>
</tr>
<tr>
<td>FEV\textsubscript{1}, %-predicted [median (range)]</td>
<td>101.5 (94.2 – 107.3)</td>
<td>115.0 (98.6 – 122.0)</td>
</tr>
<tr>
<td>FEV\textsubscript{1}/FVC, % [median (range)]</td>
<td>82.3 (80.0 – 86.3)</td>
<td>89.1 (82.2 – 98.2)*</td>
</tr>
</tbody>
</table>

\(^{1}\) Two asthmatics and 3 controls were excluded from calculation of prevalence for lack of atopy data

\(^{2}\) Statistical significance: * = p<0.05; Un-paired, two-tailed Student’s t test

\(^{3}\) Measures indicate pre-bronchodilator responses

4.4.3. Differential Blood Cell Counts

In the whole blood system, monocytes serve as the primary source of acute phase cytokines such as IL-1\(\beta\) and IL-6 (Boneberg & Hartung, 2002). Other leukocyte sub-populations, such as eosinophils, are capable of secreting such mediators, but do so at
significantly lower levels (Spencer et al., 2014). A complete blood cell count with a
differential count of leukocytes was determined for each subject’s blood donation to
control for variations in cell counts that would influence the WBA response. The
summary statistics of the differential white blood cell (WBC) counts are summarized in
Table 4.3. The asthmatic subjects were further dichotomized based on the proximity of
their residence to the highway, since subjects living within 75 m of the highway exhibited
higher relative blood monocyte counts than asthmatics in the background exposure group
or non-asthmatics in the control group. No other significant differences in WBC counts
were observed between sexes or within groups. Notably, the eosinophil counts for
controls were also high (3.9% of WBC) and 80% of these children were atopic.

Table 4.3. Differential White Blood Cell Counts

<table>
<thead>
<tr>
<th></th>
<th>Asthmatics&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Non-asthmatic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Near-Highway</td>
<td>Background</td>
</tr>
<tr>
<td></td>
<td>GM (95% CI)</td>
<td>GM (95% CI)</td>
</tr>
<tr>
<td></td>
<td>(N = 16)</td>
<td>(N = 16)</td>
</tr>
<tr>
<td>WBC (10&lt;sup&gt;3&lt;/sup&gt; cells/µl)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>7.4 (6.3 – 8.6)</td>
<td>6.8 (5.9 – 7.7)</td>
</tr>
<tr>
<td>Lymphocytes (% WBC)</td>
<td>37.3 (33.9 – 41.0)</td>
<td>35.8 (30.7 – 41.7)</td>
</tr>
<tr>
<td>Neutrophils (% WBC)</td>
<td>47.0 (42.5 – 52.1)</td>
<td>47.6 (42.5 – 53.3)</td>
</tr>
<tr>
<td>Monocytes (% WBC)</td>
<td>6.4 (5.7 – 7.3)*</td>
<td>5.1 (4.6 – 5.7)</td>
</tr>
<tr>
<td>Eosinophils (% WBC)</td>
<td>6.5 (4.8 – 8.7)</td>
<td>4.1 (2.5 – 6.7)</td>
</tr>
<tr>
<td>Basophils (% WBC)</td>
<td>0.2 (0.0 – 0.4)</td>
<td>0.2 (0.0 – 0.3)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Asthmatic participants grouped by location of the residence, in relation to highway. *Near-Highway group: residence < 75 m from highway; Background group: residence > 150 m from highway.*

<sup>2</sup> Absolute WBC counts, with sub-populations reported as relative percentage of total WBC (% WBC).

<sup>3</sup> One-way ANOVA with Dunnett’s post test: *p<0.05, with non-asthmatics as reference group.
4.4.4. WBA Cytokine Response

To determine if IL-1β response of whole blood differed by asthma status, donor blood was stimulated, ex vivo, with endotoxin at two concentrations (10 µg/ml and 100 ng/ml), and a single extract of PM$_{2.5}$ particles collected from air near the roadside in Pampas. Figure 4.3 summarizes IL-1β cytokine responses for the asthmatic and non-asthmatic groups. No difference in mean cytokine response was evident between groups following stimulation with either endotoxin or PM$_{2.5}$. Similarly, no difference in IL-1β response was observed when participants were dichotomized by sex or atopy status. The IL-1β signal from the WBA testing was normalized to donor monocyte counts. The coefficient of variation for triplicate WBA incubations of PM$_{2.5}$ samples was 10%, indicating strong reproducibility of whole blood responses to this complex stimulus.

![Figure 4.3](image.png)

Figure 4.3. No difference between asthmatic and non-asthmatic participants in WBA IL-1β response to endotoxin (Panels A & B) or PM$_{2.5}$ (Panel C)

\[^1\] Tukey Box plots with median and IQR
We next evaluated the WBA data as a function of residential proximity to the four-lane highway that splits the Pampas study area. Since asthma status had no apparent influence on IL-1β responses to endotoxin or PM$_{2.5}$, study participants were grouped by the proximity of the residence to the study area highway. Figure 4.4 summarizes the IL-1β responses for the children living within 75 m of the road, and those living 150 m or more from the road.

Figure 4.4. IL-1β responses to WBA stimulation with PM 2.5 are reduced for study participants living within 75 meters of the study area highway in Pampas

These results indicated that the pro-inflammatory potential of traffic PM$_{2.5}$ was attenuated for all subjects from residences within 75 m of the highway. In comparison to subjects who lived farther from the road (< 150 m), the peripheral blood from participants living within 75 m of the road released 50 percent less IL-1β (normalize to monocyte count) in response to stimulation with an extract of ambient PM$_{2.5}$ particles collected
from the study area. This disparity in IL-1β response was not observed when participant blood stimulated with purified endotoxin. Sensitivity analysis, in which outlier cytokine responses were excluded (based on Grubb’s test), did not significantly alter statistical relationships.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respirable PM Mass vs. IL-1β Response</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Near-highway</td>
<td>15</td>
<td>0.56</td>
<td>0.03</td>
</tr>
<tr>
<td>Background</td>
<td>15</td>
<td>0.34</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Black Carbon vs. IL-1β Response</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Near-highway</td>
<td>15</td>
<td>0.34</td>
<td>0.22</td>
</tr>
<tr>
<td>Background</td>
<td>15</td>
<td>0.24</td>
<td>0.38</td>
</tr>
<tr>
<td><strong>Endotoxin vs. IL-1β Response</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Near-highway</td>
<td>14</td>
<td>0.46</td>
<td>0.08</td>
</tr>
<tr>
<td>Background</td>
<td>12</td>
<td>0.78</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

1 Samples grouped by sampling location in relation to highway. Near-Highway group: residence < 75 m from highway; Background group: residence >150 m from highway.
Given the potential for spatial variation in the composition of the respirable PM collected for this study, we sought to investigate correlations between IL-1β cytokine response and the PM mass, BC, and endotoxin content of the sample extracts used to stimulate participant whole blood (summarized in Table 4.4). Here, we observed a modest positive correlation ($r = 0.78$, $p < 0.01$) between log-transformed participant IL-1β responses and the log-endotoxin content of respirable PM samples that were collected from background locations (distances $>150$ m). However, endotoxin and IL-1β were not correlated for participants living within 75 m of the highway ($r = 0.45$, $p = 0.08$). The particle mass concentration of the respirable PM extract and IL-1β responses were weakly associated for these individuals living near the highway ($r = 0.56$, $p < 0.05$), with association observed at background. No other significant correlations between PM samples and WBA responses were found and the exclusion of statistical outliers did not significantly alter relationships.
4.5. Discussion

Residential proximity to major highways is strongly associated with increased exposure to ambient traffic pollution in many populations (HEI, 2010). In the context of childhood asthma, the increased traffic PM exposures that occur near to highways have been associated with an elevated prevalence of asthma symptoms (McConnell et al., 2006; Venn et al., 2001), decreased lung function (Holguin et al., 2007), and high rates of allergic sensitization (Baumann et al., 2011; Morgenstern et al., 2008). However, aeroallergens do not appear to consistently explain the hazards of this PM for asthmatics (Miller & Peden, 2014). Despite the increasing mass of evidence to substantiate traffic PM exposure as a risk factor for asthma, it remains difficult to determine if asthmatics are uniquely susceptible to traffic PM-induced inflammation.

This study investigated the capacity of two distinct types of stimuli (E. coli endotoxin and traffic PM) to elicit the release of a conserved pro-inflammatory cytokine from the peripheral whole blood systems of non-asthmatic and persistent asthmatic children who live near to and far from a major highway. To our knowledge, this is the first study to employ this WBA approach for the purposes of air quality exposure assessment in asthmatic populations. The children who were enrolled in this study were recruited from homes clustered around a busy four-lane highway in the Pampas community. Half of these children lived within 75 m of the highway, while the remainder lived at background distances of 150 m or more. Study participants were recruited from these zones after preliminary sampling indicated that the BC content of ambient PM$_{2.5}$ decreased at distances beyond 100 m from this highway. Therefore, significant reductions
in traffic PM constituents were considered to occur at distances of 100 m or more from the highway.

The PM sampling that was conducted for this project focused on the respirable fraction of ambient particles, as opposed to the PM$_{2.5}$ fraction, since these samples were collected for use in the WBA. The respirable fraction, a deposition-based sampling convention, was considered to represent a more biologically representative fraction of particles and stimuli from the environment, which may be excluded by PM$_{2.5}$ sampling (Cherrie & Aitken, 1999). The air sampling for this study indicated that respirable concentrations of PM near the main highway were only 1.2 times greater than at background (27.2 µg/m$^3$ vs. 23.6 µg/m$^3$, p = 0.04). Co-located PM$_{2.5}$ samples collected from these locations showed no significant differences, with the respirable size fraction indicating that most of the particle mass was less than 2.5 µm in diameter. On average, both the ambient respirable PM and PM$_{2.5}$ concentrations in this study area exceeded the World Health Organization’s (WHO) guideline for annual average concentrations of PM$_{2.5}$ (10 µg/m$^3$) and were nearly equivalent with the World Health Organization’s guideline for maximum average 24-hour exposures to PM$_{2.5}$ (25 µg/m$^3$) (WHO, 2005).

The BC content of this PM was investigated as a traffic-related exposure variable and as a proxy for the potential of this PM to stimulate IL-1β cytokine response from the WBA. BC is often treated as an indicator of traffic pollutants (e.g. – metals, polyaromatic hydrocarbons) (Janssen et al., 2011). The estimated mass concentration of BC in ambient air near the highway was 1.5 times that of background levels (8.4 µg/m$^3$ vs. 5.8 µg/m$^3$, p <0.001). The average highway-to-background ratio of PM and BC observed in this study was in fair agreement with results of the systematic review conducted by Janssen et al.,
Moreover, the spatial relationship in BC content reflects the concentration gradients reported by Zhu et al. (2002) and Hitchins et al. (2000), who conducted studies of ultrafine PM in the vicinity of heavily trafficked roadways and freeways.

Bacterial endotoxins were a second class of constituents that were quantified in the PM samples for this study. Endotoxins are lipopolysaccharides from Gram-negative bacteria, which carry potent immunostimulatory potential in the human innate immune system (Beutler & Rietschel, 2003), and are also considered a risk factor for asthma (Doreswamy & Peden, 2011; Breysse et al., 2010). The endotoxin content of PM was measured in this study to control for its pro-inflammatory effects in the WBA. We also assessed the concentrations of respirable endotoxin in the ambient air of Pampas.

The endotoxin content of this respirable PM did not differ spatially across the study site. At an average content of 3.3 EU/mg, Pampas PM carried a relatively low burden in comparison to that of urban Baltimore City air (measured at 19 EU/mg in unpublished data from the lab of this study’s investigators). In filter eluates, the average endotoxin concentration of respirable PM samples was 0.1 EU/ml. This concentration is generally considered to be below the whole blood system’s threshold of sensitivity for endotoxin, which is reported in the range of 0.25 – 0.5 EU/ml (Hasiwa et al., 2013; Daneshian et al., 2009). However, 31 of the 32 respirable PM samples generated a significant IL-1β response in the WBA, suggesting that a non-endotoxin source of stimuli contributed to the pro-inflammatory potential of this PM.

For PM samples collected from ambient air at background distances, respirable endotoxin (but not PM mass) was modestly correlated with the WBA cytokine response ($r = 0.78$, $p < 0.01$). This testing used whole blood from participants living in the homes
where these samples were collected. The correlation between respirable endotoxin and the IL-1β signal of the WBA was not found when PM samples collected near the highway were tested ($r = 0.46, p = 0.08$). However, the IL-1β signal generated by WBA tests of near-highway PM was weakly correlated with PM mass ($r = 0.56, p = 0.03$).

Overall, the WBA analysis for this study suggested that residential proximity to the highway, but not asthma status, was most strongly associated with altered acute phase cytokine response in peripheral whole blood from these study participants. When the IL-1β signal from PM$_{2.5}$-stimulated whole blood was normalized to the participant’s blood monocyte count, it was found that IL-1β responses from subjects living near the highway were approximately one-half that of children who lived at background distances. This difference was not evident in un-adjusted IL-1β responses due to the elevated blood monocyte counts of asthmatic children who lived near the highway. No difference in mean blood monocyte counts were observed for controls in this study, although they represent a small sample size to draw conclusions from.

Although we are aware of no previous studies that have used WBA methods to investigate environmental PM exposure in asthmatic or atopic whole blood, there are a variety of WBA studies that have assessed acute phase inflammatory responses to challenge with endotoxins, fungal material, and settled dust. Bettiol and colleagues (2000) used WBA methods to assess innate and adaptive cytokine responses (TNFα, IL-6, IFNγ, and IL-4) to endotoxin and phytohaemagglutinin (PHA) exposure, using fresh whole blood from asthmatic (atopic and non-atopic) and non-asthmatic adults (healthy controls). The authors of this study reported that whole blood from both atopic and non-atopic asthmatics displayed higher background levels of IL-6 in un-stimulated blood, as
compared to non-atopic, non-asthmatic controls. Both atopic and non-atopic asthmatics were also shown to have an enhanced release of IL-6 following stimulation with endotoxin, in comparison to healthy controls. No difference in the IL-6 signal was evident between atopic and non-atopic asthmatics. Conversely, when the TNFα signal was measured in the same incubations, no differences in response to endotoxin were found between groups of participants, regardless of asthma or atopy status.

Although Bettiol and colleagues did not measure IL-1β in this study, TNFα and IL-6 also represent acute phase inflammatory cytokines that are similarly implicated in the innate immune response and carry overlapping inflammatory properties with IL-1β. The disparate trends in endotoxin-induced TNFα and IL-6 measures in this study suggest that multiple acute phase cytokines may need to be assessed to properly detect differences in individual responses to stimuli such as endotoxin. Just as endotoxin may not explain the total pro-inflammatory potential of PM, a single non-specific inflammatory cytokine may not sufficiently explain the inflammatory response to stimulation with complex environmental samples.

Sahlander et al. (2010) assessed innate immune function via WBA in individuals with chronic exposure to endotoxin and organic agricultural dusts. WBA cytokine responses (IL-6) were quantified for swine farmers and healthy controls, following ex vivo stimulation of whole blood with endotoxin and settled dust from a swine barn. In comparison to healthy controls, swine farmers displayed a dampened IL-6 signal in the WBA following stimulation with swine facility dust, but not endotoxin. Cytokine concentrations were normalized to total leukocyte counts.
Based on the endotoxin analysis of this study’s respirable PM samples, children in the Pampas community were not chronically exposed to high levels of ambient endotoxin or other biogenic constituents. However, participants living near the busy four-lane highway were exposed to slightly higher concentrations of ambient traffic PM and significantly higher concentrations of BC. These were also children who exhibited comparatively lower IL-1β responses following WBA stimulation with PM$_{2.5}$ collected from roadside air in the study area. Yet, no difference was observed for endotoxin-induced IL-1β signals for any group of study participants. It is possible that high levels of atopy in this study population played a role in mediating the IL-1β response to endotoxin and potentially reduced the heterogeneity in responses. The endotoxin concentrations used were also significantly higher than the measured endotoxin content of the PM extract. These differences in response may only be apparent at low levels of stimulation.

There are a number of study limitations that may have affected the outcomes of this study. The sample size for this study was modest, which reduced the statistical power to detect differences in IL-1β signal between groups of individuals. Participants were also predominantly atopic, and the effects of atopy on WBA cytokine response are not well established. Additionally, only a single inflammatory cytokine was tested in these incubations. As shown by Bettiol and colleagues (2000), other inflammatory cytokines may respond differently to the stimuli, particularly for complex mixtures like traffic PM.

Testing these whole blood incubations for a broad panel of cytokines would serve to better characterize immune responses and provide insight into both innate and adaptive responses. Collecting air samples and participant blood at multiple time points would also enhance our understanding for the immunologic effects of traffic PM exposures in these.
individuals. Wouters et al. (2002) and Spreitzer et al. (2007) have shown that greater variations in cytokine production occur between individuals (rather than within individuals). However, the findings of this study would be more robust if repeat measures were obtained, since the composition of PM may change over time as well.

Lastly, this study focused on the pro-inflammatory effects of respirable PM and PM$_{2.5}$. Other relevant pro-inflammatory stimuli may be carried by larger non-respirable PM, which could also serve to stimulate bronchial inflammation and exacerbate asthma. Notably, the highest endotoxin signals have often been associated with the larger diameter of aerosolized particles. The thoracic fraction of PM, which still deposits in the conducting airways, may carry a different pro-inflammatory hazard for these children. The concentrations of this PM, in relation to the highway, may also differ significantly since the larger particles (re-suspended by traffic activity) settle faster than those of the respirable diameter.
4.6. Conclusions

In conclusion, the findings of this study provide exploratory evidence of altered systemic innate immune function in asthmatic and non-asthmatic children living near this four-lane highway in the Pampas community. Study participants who lived within 75 m of the highway where exposed to ambient concentrations of traffic PM and BC that were elevated above background levels measured at distance of 150 m or more from the highway. The WBA analysis did not reveal obvious signs of an immunologic susceptibility to traffic PM or endotoxin for asthmatic children. The results of this study contribute to the existing evidence-base that suggests that close residential proximity to major highways is a risk factor in the immunologic health of individuals with chronic exposure to traffic pollution.

Acknowledgements

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Chapter 5
5.1 Summary

The research presented in this dissertation focuses on the use of human WBA methods to assess the capacity of environmental PM to stimulate inflammation. Taken together, the results of the two field studies demonstrate the flexibility of the human WBA to provide insight into both the pro-inflammatory characteristics of complex environmental stimuli and individual immune responses to a variety of different stimuli. The integrative capacity of the WBA is an attractive element for exposure assessment purposes as it is capable of assessing exposures in a manner that is similar to exposures in nature. The assay is capable of assessing mixtures and it also mimics the acute phase cytokine response that results from the interplay between environmental stimuli and the human innate immunity.

5.1.1. Broiler Poultry House PM

The study of the broiler poultry house environment, presented an opportunity to use the WBA as a standardized method for contrasting the pro-inflammatory potential of different environmental samples. While WBA methods have traditionally been confounded by differences in donor whole blood systems, the use of pooled, cryopreserved whole blood allowed for a variety of PM samples to be collected at different time points and tested within a standardized whole blood system.

The cryopreserved WBA provided evidence that the quantification of endotoxin, alone, does not capture the total pro-inflammatory potential of PM from the broiler poultry house environment. This was not surprising, given the diverse microbial communities in the poultry houses. The WBA testing indicated that as much as half of the pro-inflammatory signal is mediated by non-endotoxin stimuli in the PM matrix. This
WBA analysis also allowed for the investigation of insoluble stimuli, which are discarded in the LAL testing process, and indicated that they account for as much 50 percent of the total IL-1\(\beta\) response observed in the analysis. Further research needs to be conducted to determine the composition of stimuli in both the “non-endotoxin” and “insoluble” fractions of this PM, such that we can better understand which biogenic sources of PM are the most important in these environments.

There is also a need to incorporate WBA methods into human exposure studies to better evaluate the exposure-response relationships between PM inhalation and the variety of non-infectious respiratory health effects that are thought to be driven by biogenic constituents. This is important since the LAL-measured endotoxin content of organic dusts and PM may not reliably predict the occurrence or severity of these health effects. Therefore, it is critical to holistically determine the pro-inflammatory potential of PM and identify which size fractions carry the most hazardous burden of stimuli. In this study, when the LAL and WBA signals were normalized to PM mass, the larger particle fractions consistently carried the greatest burden of endotoxin and the highest capacity to elicit the release of IL-1\(\beta\) from cryopreserved whole blood. However, the threshold for eliciting airway inflammation in different zones of the respiratory tract are not well understood and thus it may require only a low burden of stimuli in the small particle sizes to represent a hazard for the sensitive small airways.

Overall, the cryopreserved WBA shows promise as an exposure assessment tool. In the future the WBA method needs to be optimized to determine how to most efficiently test PM samples (e.g. – concentrations of extracts, whole filters, bulk PM) and how to best capture the relevant cytokine responses. Currently the air filter extraction
process contains many critical points at which stimuli may either be lost or introduced in the sample. For WBA methods to gain added traction in exposure science, they will need to be further standardized, such that results are more easily interpreted and carry enhanced generalizability.

5.1.2. Traffic PM & Asthma

The use of WBA methods in the study presented a very different challenge from that of the poultry house study. While the results of the WBA analysis did not yield significant insight into immunologic susceptibilities of asthmatic children who were exposed to traffic PM, they did provide evidence that a conserved element of systemic immune function differs between children living near and far from the major highway in the study site. This finding warrants further investigation as it suggests that innate immune response to constituents in traffic-related PM is attenuated for individuals with chronic exposure. Since acute phase inflammation is critical component of both front line of defenses against infection and the repair of tissue injury, a depressed response to innate immune stimulation may confer a susceptibility to immunologic challenges from the environment that are accompanied by traffic-related PM constituents. However, we are careful to also note that peripheral whole blood from all study subjects responded similarly to challenge with purified endotoxin. Thus, innate response to bacterial PAMPs does not appear to be similarly affected.

To add to this research, a larger panel of cytokines needs to be assessed in whole blood incubations from these children, such that the innate and adaptive immune responses can be better characterized and contrasted between individuals. While IL-1β has proven to be a suitable marker of inflammatory response to stimulation in a variety of
endotoxin and non-endotoxin stimuli, it is unlikely to summarize the full acute phase response to environmental PM exposures. WBA incubations provide the opportunity to extract a wealth of immunologic data. A concerted effort should be made to extract more comprehensive biologic insight and incorporate high throughput approaches towards assessing these PM exposures.

The WBA also serves as a field applicable immunologic assay that allowed our study team to conduct a significant amount of this work in a low-resource field in the study area. This simplified the logistics of the study, allowed for the use of commercially available reagents and equipment, and did not require extensive handling or processing of blood for the test. The results of the study provided us with a snapshot of the innate immune function of these asthmatic and non-asthmatic Peruvian children that could not have been achieved with another assay under these conditions.
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Curriculum Vitae

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Education & Training

2015   PhD (ABD, expected January 2015)                      The Johns Hopkins University
       Environmental Health Sciences (GPA: 3.84/4.0)          Baltimore, Maryland
       Thesis: Whole Blood Assay Analysis of Immunostimulatory Constituents in Environmental
       Particulate Matter
       Advisor: Patrick N. Breysse, PhD, CIH

2009   MEng                                           Cornell University
       Engineering Management (GPA: 3.89/4.0)                  Ithaca, New York

2005   B.S.                                           University of Alaska Anchorage (UAA)
       Civil Engineering (GPA: 3.61/4.0)                      Anchorage, Alaska

Professional Registrations & Certificates

• Registered Professional Engineer (Civil) - Utah #7752078-2202
• The Johns Hopkins University Risk Sciences & Public Policy Certificate

Research Projects

2014 - present  Microbiome Analysis of Broiler Poultry House Particulate Matter
                 PI: Patrick N. Breysse; Co-PI: Alan Scott
                 Comprehensive investigation of bacterial and fungal microbiome within broiler
                 poultry house litter, feces, feed, and airborne particulate matter (PM). Next-generation
                 DNA sequencing methods used for a metagenomic analysis of microbial diversity.

2013 - present  Susceptibility to Asthma and Air Pollution in Peru: Traffic PM Exposure and
                 Inflammatory Mediator Production in the Human Whole Blood System
                 PI: Nadia Hansel
                 Study of asthmatic Peruvian children in Lima, using human whole blood assay
                 methods to investigate each participant’s immunologic response patterns to standard
                 endotoxin stimuli and respirable traffic PM sampled from the local environment.

2012 – 2014  Whole Blood Assay Assessment of Endotoxin and Non-Endotoxin Inflammatory
             Stimuli in Organic Particulate Matter
             PI: Patrick N. Breysse
             Development and use of cryopreserved human whole blood assay methods to
             characterize and quantify the pro-inflammatory potential of respirable, thoracic, and
             inhalable PM samples from animal housing facilities (broiler poultry and dairy cow)
             and ambient urban Baltimore City.
2011 - 2012  Compositional Analysis of Particulate Matter from Rural Indian Cookstoves
PI: Patrick N. Breysse
Sampling of particulate matter from kitchen air in rural Indian homes that utilize biomass cookstoves, and analysis of samples for metals and PAH content.

Professional Experience

2005 – 2010   CH2M HILL                                    Anchorage, Alaska - Salt Lake
City, Utah
Role: Civil Engineer in Transportation Business Group

Academic Teaching Experience

2013   Teaching Assistant  Johns Hopkins Bloomberg School of Public Health
Course: Environmental & Occupational Health, Law, & Policy

2012   Teaching Assistant  Johns Hopkins Bloomberg School of Public Health
Course: Food Production, Public Health, & the Environment

2009   Teaching Assistant  Cornell University
Course: Risk Analysis and Management

2008   Teaching Assistant  Cornell University
Course: Construction Planning and Operation

Peer Review Publications


Posters & Presentations


### Applicable Coursework

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### Academic Awards

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